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RELATION BETWEEN NUTRITIONAL STATUS, THE OUTCOME OF INFECTION AND IMMUNE RESPONSES

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Relation between nutritional status, the outcome of infection and immune responses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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A las oportunidades / To opportunities

ABSTRACT

A causal interaction between nutrition and the outcome of different infections has been known since long time ago. Moreover, responses of human beings to infection are diverse and demarcated by immunity. A deficient and unappropriated nutrition allows to a deficient immune response. The dynamic interconnection between nutrition, immunity and the outcome of infection creates a cycle. If one of these states is altered, the proper balance of a healthy system will varnish. The purpose of this thesis is to introduce studies that reflect this interaction by exploring both, epidemiological and experimental settings.

The first cross-sectional, epidemiological study showed a considerable prevalence of overweight and obesity in school children from Caranavi, a community that represented the Bolivian Lowlands. As for the Highlands, Taraco community remained opposite, with higher prevalence of undernourishment and even stunting, while overweight and obesity were not that prevalent. Remarkably, anemia was found highly prevalent in children from Taraco, compared to lower numbers in Caranavi. More than half of both populations showed vitamin D insufficiency. This study showed increased anaemia, nutritional deficiencies, and indications of poor hygienic conditions in highlands compared with lowlands.

Intestinal nematodes modulate immune responses in the context of secondary infection and vaccination. Several mechanisms have been proposed for a possible suppressive effect. In the second study, it was shown that chronic nematode infection leads to reduced peripheral responses to vaccination because of a generalized reduction in the available responsive lymphocyte pool. In detail, it was shown that superficial skin-draining lymph nodes (LNs) in infected mice with the intestinal nematode *Heligmosomoides polygyrus*, have an altered lymphocyte composition and less cellularity than uninfected mice upon subsequent BCG infection in the skin. Moreover, B cells and T cells were also lower in skin draining LN. Notably, de-worming and time improved lymphocyte cellularity in LNs and restored *Bacillus Calmette-Guerin* (BCG) responses in the draining LN of the footpad injection site, concluding that chronic nematode infection leads to a paucity of lymphocytes in distal lymph nodes that reduces the efficacy of peripheral immune responses.

Obesity can lead to diabetes. Moreover, diabetes increases the risk of developing tuberculosis (TB), but the underlying mechanisms in the tuberculosis-diabetes (TB-DM) co-morbidity are not well defined. This study analyzed the susceptibility to *Mycobacterium tuberculosis* (Mtb) in a diabetic mice model and focused in the role of HIF-1 α , a main regulator of metabolic and inflammatory responses, in the outcome of Mtb infection under hyperglycemia and diabetic models. It was observed that mycobacterial infection of macrophages (BMM) or mice increased the expression of HIF-1 α -regulated *glut1* and *vegfa* genes. Interestingly, incubation of BMM with deferoxamine (DFO), a HIF-1 α stabilizer, increased levels of HIF-1 α -regulated immune and metabolic transcripts in both mycobacteria-infected and uninfected BMM. Moreover, *M. tuberculosis* load was reduced in DFO-incubated BMM. Hyperglycemic conditions reduced the expression of *glut1*, *vegfa*, *inos* and *illb* mRNA in mycobacteria infected BMM and DFO treatment reversed this effect. In line with this, *M. tuberculosis* levels

were higher, and HIF-1 α - responses reduced in lungs from db/db mice. DFO-treated mice had increased levels of HIF-1 α -regulated transcripts in infected organs, and reduced *M. tuberculosis* load in lungs. These findings suggest that an increase of HIF-1 α function can improve the control of infection with *M. tuberculosis* during DM.

Altogether, these studies, although diverse, support the importance of the link between nutrition, immune responses and the outcome of two different infections.

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LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End Products
BCG	Bacillus Calmette-Guerin
BMI	Body Mass Index
BMM	Bone Marrow-Derived Macrophages
DAMPS	Damaged-Associated Molecular Patterns
DM	Diabetes Mellitus
EPT	Extrapulmonar Tuberculosis
FFA	Free Fatty Acids
GDM	Gestational Diabetes Mellitus
H. polygyrus	Heligmosomoides polygyrus
HbA1c	Glycated Hemoglobin
HDL	High Density Lipoprotein
HFD	High Fat Diet
HIF	Hypoxia Inducible Factor
IFN	Interferon
ILCs	Innate Lymphoid Cells
iNOS	Inducible Nitric Oxide Synthase
L3s	Stage 3 Larvae
LPS	Lipopolysaccharide
M. bovis	Mycobacterium bovis
MAPK	Mitogen Activated Protein Kinases
MDR	Multidrug Resistance
MGO	Methylglyoxal
MHC	Major Histocompatibility Complex
Mtb	Mycobacterium Tuberculosis

NCD	Non-Communicable Diseases
NF- κ B	Nuclear Factor- κ B
NO	Nitric Oxide
PAMPS	Pathogen-Associated Molecular Patterns
RAG	Recombination Activating Gene
ROS	Reactive Oxygen Species
RR	Rifampicin Resistant
RSV	Respiratory Syncytial Virus
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TB	Tuberculosis
TB-DM	Tuberculosis-Diabetes Comorbidity
TGF- β	Transforming Growth Factor B
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
TSLP	Thymic Stromal Lymphopoietin
UNICEF	United Nations International Children's Emergency Fund
WHO	Wild Type
WT	World Health Organization

1 INTRODUCTION

1.1 NUTRITION AND HEALTH

Nutrition is the set of integrated processes by which cells, organs and the whole body acquire the energy and nutrients for normal structure and function. It also relates to the capacity of the body to transform the substrates and cofactors necessary for metabolism. Life-maintaining chemical reactions, result in the build-up of proteins, lipids, nucleic acids and carbohydrates ¹. In simpler words, the World Health Organization (WHO) defines nutrition as the intake of food considered in relation to the body's dietary needs ².

A balanced nutritional state responds to tightly associated external and internal factors. Externally, it is related to the environment and society, for example, the quality of consumed food. The internal factors are mostly related with the functioning of cells, organs and tissues through chemical, physiological and metabolic mechanisms ³. The control and maintenance of good nutrition in the world population has always been of special concern, mainly because of the negative consequences its absence has for global health.

1.1.1. Malnutrition: Under- and Overweight

The term malnutrition reflects an excess or imbalance of a wide range of nutrients, resulting in a measurable adverse effect on body composition, function and clinical outcome. There is still a misleading use of malnourished as undernourished only. However, when referring to malnourished individuals, it is important to be aware that they can be under- or over-nourished.

Malnutrition is responsible for more ill health among people than any other cause ⁴. Most forms of malnutrition are associated with different types of diseases and higher levels of mortality in comparison to the same diseases seen in well-nourished patients. The WHO exposes alarming numbers regarding the actual nutritional state nowadays. Around 1.9 billion adults are overweight or obese, while 462 million are underweight, 52 million children under 5 years of age are wasted, 17 million are severely wasted and 155 million are stunted, while 41 million are overweight or obese (definition of nutritional states farther down). Mortality is still a significant consequence of this nutritional imbalance, and around 45% of deaths among children under 5 years of age are linked to malnourishment. These deaths mostly occur in low- and middle-income countries. At the same time, in these same countries, rates of childhood overweight and obesity are rising ⁵. The combination of these two extremes of malnutrition in a same place is known as the double-burden effect.

There are several factors that play a role to define a nutritional status in a certain population. For instance, access to water, sanitation and hygiene linked to education and incomes are all essential. Moreover, the quality of diets is vital. Poor diets are the second-leading risk factor for deaths globally, accounting for 18.8% of all deaths, of which 50% are due to cardiovascular disease ⁶. Both overweight and underweight severely affect immune functions severely, leading to an increased susceptibility to severe infections. Undernutrition and overweight/obesity are

two broad groups that include more specific malnutrition subcategories. The classification below is adapted from The Global Nutrition Report 2018 - UNICEF ⁴.

Undernutrition

It is important to consider the age when classifying undernutrition, because all children under five years are in an important and intensive process of growing, learning and developing brain skills.

- i. Stunting in children under five: Refers to a form of growth failure which starts already in prenatal stages and develops over a long period of time in children under five years of age with limited access to food, health and care. Stunting is also known as ‘chronic undernutrition’, although this is only one of its causes. Stunting is associated with, and often causes cognitive impairments such as delayed motor development, impaired brain function and poor school performance.
- ii. Wasting in children under five: Children who are thin for their height because of acute food shortages or disease. Also known as ‘acute malnutrition’, wasting means a rapid deterioration in nutritional status over a short period of time in children under five years of age. The risk of dying is higher in these children. Compared to stunting, wasting children might be heavily underweight but their height is similar as compared to median of a population that has no shortage in nutrition.
- iii. Underweight in the population that is more than five years old is usually measured by body mass index (BMI), a simple index of weight-for-height (weight in kg/height in m²). If the BMI is less than 18.5 indicates underweight in adult populations while a BMI less than 17.0 indicates moderate to severe thinness. A BMI less than 16.0 is known to be associated with a markedly increased risk for ill health, poor physical performance, lethargy and even death; this cut-off point is therefore a valid extreme limit.
- iv. Micronutrient deficiencies: Undernutrition also includes an important group of people with micronutrient deficiencies. This stands for a poor nutritional status caused by a lack of intake, absorption or use of one or more vitamins or minerals needed for essential physiological functions, growth and development. Several micronutrients that remain issues globally include iron, zinc, vitamin A, D, folate and iodine, as they are the most difficult to satisfy without diverse diets. One general indicator of micronutrient deficiencies is anemia, as this syndrome is caused by the deficiency of many of them, and its effects are exacerbated by several diseases

Overweight and Obesity

An abnormal or excessive accumulation of body fat impairs health. Overweight and obesity can also be measured by BMI. In adults, overweight is defined as a BMI of 25 or more, whereas obesity is a BMI of 30 or more ⁷. In children, BMI is adjusted to age to measure malnutrition. In this line, the BMI/age indicator is the most accurate anthropometric measurement for children. Overweight and obesity cause morbidity and mortality through multiple effects on nearly all human functions and are major causes of many non-communicable diseases (NCDs), including non-insulin-dependent diabetes mellitus, coronary heart disease and stroke. They also increase the risks for several types of cancer, gallbladder disease, musculoskeletal disorders, respiratory symptoms and the susceptibility to several infections ⁸.

1.1.2. Nutrition and Infections/Immunity

Malnutrition has a clear but, in many cases, not well-defined role in the regulation of immune responses. The association of nutritional status with the risk and course of infections, and the nutrition during infection are important topics of research nowadays. Experimental and pre-clinical studies highlighted the changes in pathogen-host interactions under malnutrition and have also elucidated how these alterations impact the host's antimicrobial immune functions, the resistance to infection as well as the pathogen's proliferation and pathogenicity ⁹.

Regarding the influence of nutrition on the risk of infections, the focus has frequently been on underweight conditions rather than on obesity. There is an association between anthropometric parameters of undernourishment and higher severity of infections. For instance, viral diseases as influenza and RSV, bacterial infections as tuberculosis (TB) and colonization by *Streptococcus* and parasites including *Schistosomiasis* and *Amebiasis* have all been associated with undernourishment at the population levels ¹⁰. Nutritional intervention has been performed clinically in TB patients, but the improvement evidence is still weak ¹¹. Cross-sectional community surveys revealed increased prevalence of intestinal parasitic infections in children between 6 months to 15 years with low BMI ¹². Moreover, a prospective study showed stunted children aged 6 weeks to 1 year and HIV-infected with an increased incidence of malaria infection ¹³.

Underweight children are not only at higher infection risk in developing countries. In USA, a clinical-chart review revealed that underweight children were more frequently admitted to emergency units with respiratory infections than normal-weight, overweight or obese children ¹⁴. With an increase understanding of severe childhood malnutrition and the role of oxidative stress, the primary role formerly ascribed to protein deficiency is no longer accurate, neither concerning the condition in general, nor to the impact of malnutrition on susceptibility to infections ¹⁵. Moreover, the insufficient intake of carbohydrates and lipids has an important effect on innate immune responses. For instance, dietary restriction resulted in reduced neutrophil trafficking to sites of inflammation, due to a reduction in integrin expression and chemokine production ¹⁶. Moreover, the necessity of glucose and glutamine in macrophages and neutrophils is evident, they rely on a withdrawal of these to metabolize and produce

cytokines and phagocytosis. Moreover, certain fatty acids also modulate macrophage and neutrophil resources ^{17, 18}.

When it comes to obesity, less evidence of its impact on the immune system is found. Obesity has been linked with increased incidence of infectious diseases, including periodontal infections, influenza, bacterial pneumonia, nosocomial and surgical site infections ^{19,20}. In a cross-sectional study performed in a Dutch birth cohort, obesity in 8-year-old children was associated with more than 5-fold increased bronchitis rates and also raised use of antibiotics ²¹. Similarly, in an inpatient US database of participants aged 2–20 years the risk of urinary tract infections was increased in obese females but not in obese males. Obesity was defined by >95th percentile of weight in this study ²².

Several mechanisms involved in the increased susceptibility to infections during obesity have been suggested. For instance, susceptibility to infections in obesity might be related to decreased availability of arginine and glutamine, resulting in decreased tumor necrosis factor (TNF α) production, as shown in obese rats ²³. Moreover, another experimental model showed that obese mice exhibited an exaggerated proinflammatory and thrombogenic responses under the same stimuli as control mice. The connection of proinflammatory states of obesity with the risk of infection has not been precisely determined, but leptin seems to have an important role in the immune response. Leptin deficiency has been associated with susceptibility to infections in animals as well as in human beings ²⁴.

1.1.3. Nutrition in Children from Bolivia: Situation in High and Lowlands

Latin America and the Caribbean are still regions where hunger and malnourishment are an important health problem. The best way to address this scourge is by linking food security, sustainability, agriculture and health. Around 5.5% of the regional population are still undernourished, and 11.3% of children under 5 years old face stunting while 7.2% are overweight ²⁵.

Bolivia is one of the most poorly developed Latin American countries and has the highest prevalence of undernutrition among the Andes. Data from 2009 states that 27% of children are stunted, 14% are underweight and 3% are wasted ²⁶. Due to the influence of globalization, Bolivia is now in a nutritional transition; because of that the local food consumption is competing with the incorporation of “junk food” that is usually high in calories from sugar or fat, with little dietary fiber, protein, vitamins or minerals. There is limited knowledge regarding the actual situation of nutritional status in children that live in small districts in Bolivia, but, it is known, that rural areas from highlands have limited availability of nutrients because of the environmental conditions, where some foods like vegetables and others are hard to grow, while this is not the case in the Lowlands.

Highlands

One-third of Bolivia is located within the Andean mountain range. The altiplanic highlands are known for their fragile ecosystem and high poverty level. Altitude reaches more than 4000 masl (meters above sea level), and only few areas in the world develop agriculture in this harsh environment. Altogether, this explains why the nutritional intake in Bolivian highlands is lower than in the tropical valleys. Previous studies mentioned the frequent use of potatoes and other tubers as well as some crops as quinoa in the Andean diet. A systematic review made in different Andean highlands of Latin America including Bolivia, showed a low average intake of dietary fat, iron, zinc, calcium, vitamin A, folate and vitamin B12 ²⁷. A study that measured the dietary quality index based on FAO standards showed that children's diet were adequate for all nutrients except calories, calcium, niacin and thiamin. Low oxygen pressure in the highlands also plays an important role, recumbent length gain was found to be slower in high altitude infants in the early months of life compared to children from the lowlands. Children who had lived all their lives at high altitude were found to be smaller in terms of general body size than those who had spent the shortest amount of time at high altitude ²⁸. Currently, only few studies have focused in children nutritional status in the Bolivian highlands.

Lowlands

The term "Lowlands" is extensive and conforms several regions in Bolivia, where sub-tropical valleys at 600 to 800 masl are also included. Due to less harsh conditions that allow agriculture and cattle farming to establish, nutrients are much more diverse and easily available. A variety of local fruits and vegetables is accessible for the population, and the intake of meat, chicken and fish is more frequent. These conditions are of considerable help to improve children's growth when compared to highlands. Although topographic conditions, like altitude, are important for growth development in children, other socioeconomic factors must also be considered. Socio-economic and urban development go together in children's health and growth. This comprises the changes of traditional habits of food consumption and education.

1.2. INTestinal Nematode Infections and Immunity

Parasitic worm infections are prevalent in many developing countries where hygienic conditions are poor and rudimentary. This situation facilitates the maintenance of parasite-cycle and the risk constant re-infections of individuals.

It has been estimated that one of fifth of all humans are hosting one or more species of gastrointestinal nematodes ²⁹. The 2016 Global Burden of Disease study indicated that currently 800 million of individuals are likely to be infected worldwide with *A. lumbricoides*, 451 million with hookworm, 435 million with *Tricuriasis* and 190 million with *Schistosomiasis* ³⁰. The health impact of intestinal nematode infections is the significant morbidity that they cause, because they barely produce severe pathology or mortality. Infected people, mostly children, will show symptoms ranging from different intensities of abdominal pain, diarrhea, mild to moderate anemia, and long term stunted growth with impaired cognitive development. In most endemic areas, the presence of helminth co-infection with diseases like TB and HIV are of concern. They cause a significant host resistance reduction to the infecting as well as other microorganisms causing diseases. Moreover, helminth infections have been found to affect vaccine efficacy and impair immune responses to vaccines against TB and tetanus ³¹.

However, some intestinal parasitic infections may come with benefits. For instance, an excessive cleanliness could alter the balance of the immune system without any parasite exposure, resulting in reactions to harmless environmental molecules, food antigens and others. In animal models, immunological disorders like asthma, allergy or inflammatory bowel disease have all found to be reduced in the presence of intestinal worm infections ³². In humans, this theory has gained some support from epidemiological studies. This inverse correlation between intestinal microorganisms and immune-mediated diseases is known as the hygiene hypothesis ³³.

1.2.1. *Heligmosomoides polygyrus* Infection: A Model of Murine Helminthiasis

Heligmosomoides polygyrus (Hp) is a natural intestinal parasite of mice. This parasite was first introduced in the lab by Spurlock in 1943 as an experimental infection in laboratory mice ³⁴. Nowadays, *H. polygyrus* is the most widely used model to study the impact of gastrointestinal nematode infection for immunological, toxicological and pharmacological studies. There are several characteristics that make this worm a very useful and efficient model. The infection is limited to the intestines and can be established and maintained as a chronic infection. The pathology and morbidity caused by the worm, even using high doses of larvae, is limited making *H. polygyrus* suitable for studying trace susceptibility as well as resistance to intestinal worm infection over time and in combination with other pathologies and infections.

The life cycle of *H. polygyrus* starts with a brief histotrophic larval phase in the intestinal mucosa around 24 h after oral gavaging of mice with infective L3 larvae. Usually a strong inflammatory response and the formation of a structure called “granulomata” at the site of encystment takes place during this phase. These granulomas mainly consist of macrophages

and neutrophils, but the number and frequency of granulomas depends on the resistance phenotype of mouse strains, the most resistant genotypes will develop greater granuloma numbers³⁵. Around 10 days later, a longer adult luminal phase will take place in the proximal third of the small intestine. The adult worms twirl around the small intestine villi to secure themselves, to mate and start eggs production. The eggs hatch and undergo two molts to become infective stage 3 Larvae (L3s) to continue the life cycle³⁶.

1.2.2 Immune Responses to Intestinal Nematodes: *Heligmosomoides polygyrus*: Th2/Regulatory Responses

Helminths are successful organisms. They develop chronic infections that without therapeutic intervention can last a lifetime in the host. In this parasite-host interaction, an active immunomodulation takes place, creating frequently a muted immune response in which the host adapts and tolerates the invader.

Innate immune responses are initiated by danger signals. Intestinal helminths introduce damaged-associated molecular patterns DAMPS and pathogen-associated molecular patterns PAMPS which elicit surface interactions. The activation of epithelial release cytokines like thymic stromal lymphopoietin (TSLP), IL-25 and IL-33³⁷. These cytokines promote the proliferation of innate lymphoid cells type 2 (ILC2s) that together with other cells in turn secrete IL-2, IL-4, IL-5, IL-9 and IL-13³⁸. Of importance, an increased mucus secretion in goblet cells will be induced by IL-13. To prevent recognition or limit the effect of recognition by the innate immune system the worm release molecules which block toll-like receptor (TLR) ligand-induced dendritic cell and macrophage activation³⁹.

Adaptive immunity against intestinal nematode infections are characterized by a strong Th2 type response which in chronic infections is accompanied by a regulatory response. The Th2 response can be divided in three different phases: First the production of typical Th2 cytokines like IL-4, IL-5, IL-9, IL-10 and IL-13 by T cells; then the induction of IgG1, IgG4 and IgE by B cells; and last, an expansion of eosinophils and alternatively activated macrophages and immunoregulatory monocytes⁴⁰. Th2 responses are important to promote worm expulsion. For instance, IL-4 and IL-13, which promote mucus production and smooth muscle contractions in the upper intestine are of particular importance⁴¹. IL-4 and IL-13 may have compensatory function and recent studies showed that IL-4 null mice can expel the gastrointestinal nematode *Nippostrongylus brasiliensis*⁴². However, when IL-13 was investigated, IL-13 null mice animals failed to clear *N. brasiliensis* infections effectively and IL-13 was shown to be an important link to the production of intestinal mucus, which therefore facilitates worm expulsion⁴³. After expulsion, a resolution stage will take place with a solved inflammation and tissue reparation. This step will also be partly orchestrated by type 2 cytokines, but it will also include macrophages and eosinophils which will be involved in wound healing and tissue generation⁴⁴. Eosinophils are also responsible for the generation of IgA-secreting plasma cells in the small intestine via IL-1 β production⁴⁵.

In addition, there is consistent evidence about the induction of regulatory T cells (Treg) during different helminth infections, mostly at chronic stages. In chronic infections, a hypo-responsive state will evolve to reduce the damage of the immune response and control the balance. This state will be favorable both for the long-term survival of the worms, as for the host protective immune responses.

The expression of Foxp3, the IL-2R α chain (CD25) and the production of the anti-inflammatory cytokines IL-10 and TGF- β are characteristic of Tregs. An important expansion of CD4⁺ CD25⁺ cell numbers and Foxp3, together with high expression of IL-10 and TGF- β occurred in draining mesenteric lymph nodes (mLN) during a chronic *H. polygyrus* infection⁴⁶. An effective suppression of Tregs against Th2 immunity was demonstrated in *H. polygyrus*-infected mice, facilitating persistent worm infection⁴⁷. But Tregs also play an important suppressive role, mostly at later stages of infection. For instance, Grainger et al showed an inhibition of TGF- β signaling that reduced the worm burden and resulted in an increase of Th2 responses⁴⁸. Moreover, transfer of Tregs (CD25 surface) from *H. polygyrus* infected mice gave protection from airway allergy to sensitized mice³⁶. An interesting immunoregulatory cytokine for *H. polygyrus* is TGF- β . In chronic infection of C57BL/6 mice, plasma TGF- β levels rise threefold over 30 days, but return to baseline within a week of curative drug treatment⁴⁹.

An important issue to solve regarding Tregs is if the expansion occurs within induced (adaptive) or natural (thymic) Treg types. During *H. polygyrus* infection, pre-existing natural Tregs expressing the marker “Helios” were found to be the first to expand after infection, while “Helios-negative” (i.e. adaptive) populations only appeared late and only remained a numerical minority of the total Treg compartment throughout the infection⁵⁰.

1.3. DIABETES AND TUBERCULOSIS

1.3.1. Diabetes mellitus

Diabetes mellitus (DM) is the denomination of a set of metabolic diseases that are generally defined by the presence of chronic hyperglycemia. The term “mellitus” actually means “honey sweet” and was added in 1675 by Thomas Willis because of the sweetness in urine and blood from patients ⁵¹.

Currently there are four types of Diabetes:

1. Type 1 DM (T1DM) which is caused by an autoimmune destruction of β cells in the pancreatic islets, leading to complete insulin deficiency.
2. Type 2 DM (T2DM) that is characterized by a progressive loss of β -cell insulin secretion, commonly following the development of insulin resistance.
3. Gestational diabetes mellitus (GDM), usually diagnosed in the second or third trimester and was not evident prior to gestation.
4. Specific types of diabetes due to other causes like, monogenic diabetes syndromes (neonatal and maturity-onset diabetes of the young), exocrine pancreas diseases such as pancreatitis, and drug-induced diabetes ⁵².

Type 2 Diabetes mellitus (T2DM) is the most frequent type of Diabetes and it has become an important global health problem that could aggravate with time. According to the International Diabetes Federation, 382 million of adults aged 20-70 years worldwide had type 2 DM in 2013, and 80% of this population were from low and middle-income countries with an expected rise to 592 million by 2035. Moreover, in 2017 around 4 millions of people died because of type 2 DM ⁵³.

Above all the people with DM, only 10-15% have Type 1 Diabetes mellitus. However, T1DM is the most common type in children globally, with an estimated number of 90,000 new diagnosed children every year ⁵⁴.

Despite that hyperglycemia is a common characteristic in all types of DM, the pathophysiology will differ on some aspects. Hyperglycemia is the presence of high glucose levels circulating in the blood due to defects in insulin levels or signaling. Insulin is a peptide hormone produced by the β -cells of the pancreatic islets of Langerhans. Its main function is to maintain normal blood glucose levels mainly by facilitating cellular glucose uptake and regulating the metabolism of carbohydrates, lipids, and proteins ⁵⁵.

In T2DM, the defects of insulin are related with an impaired insulin signaling and a low production of insulin ⁵⁶. Insulin resistance in muscle and liver cells results in a decreased glucose uptake and an increased hepatic glucose production that coexists with a defect of the pancreatic β -cells to produce enough insulin ⁵⁷. Moreover, insulin scarcity will increase glucagon production by the α -cells in the pancreas, also contributing to excessive glucose

production in the liver ⁵⁸. A simplified scheme of insulin alteration in diabetes is described in Figure 1.

The effect of insulin resistance in adipocytes will result in accelerated lipolysis and increased plasma free fatty acid (FFA) levels that will aggravate more the insulin resistance in muscle and liver ⁵⁹.

The complications of T2DM are related both with the severity and duration of high glucose levels in the blood and will also be evident by damage in the microvasculature ⁶⁰. Several are the pathways that contribute to this damage, of importance are the increased formation of advanced glycation end products (AGEs) and increased intracellular reactive oxygen species ⁶¹. Damage in a micro- and macrovascular level will increase the risk of cardiovascular risk factors and activation of inflammatory pathways ⁶². Macrovascular complications include stroke and coronary artery disease while nephropathy and retinopathy are microvascular complications of diabetes ⁶³.

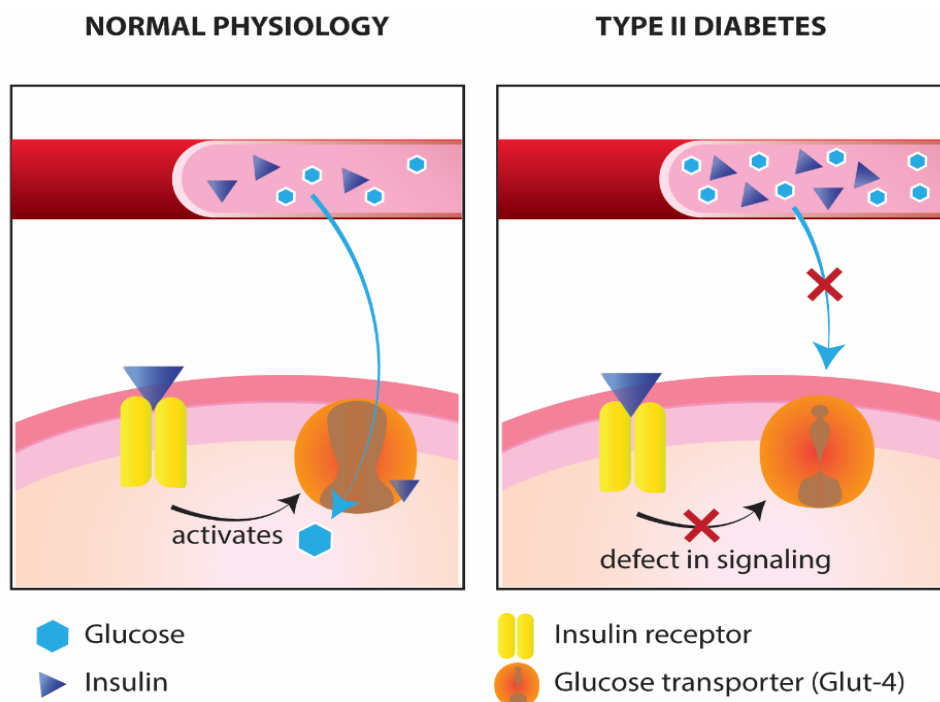


Figure 1. Defect on insulin signalling in T2DM.

1.3.2. Obesity as a risk factor for diabetes

Hypertriglyceridemia, hypertension, visceral obesity, low high-density lipoprotein (HDL) levels together with high fasting plasma glucose will conform a cluster of conditions that increase the risk of T2DM and are also associated with stroke and heart disease ⁶⁴.

Central obesity is sign and cause of the metabolic syndrome. Increasing adiposity, often reflected in high waist circumference, may both result from and contribute to insulin resistance. Obesity is an important and very prevalent global health problem. Cases had tripled since 1975, and in 2016, around 39% of adults globally (18 years old or more) were overweight, and 13%

were obese. The situation in children is not better, an alarming number of 41 million children under the age of 5 were overweight or obese in 2016, and 340 million children and adolescents (5 to 19 years old) were overweight or obese the same year ⁶⁵.

The molecular mechanisms that link obesity with DM are mainly related with insulin resistance ⁶⁶. Several mechanisms are being studied to understand the predisposition, but maybe the most important ones are: the increased production of adipokines/cytokines like tumour necrosis factor- α (TNF), IL-6 and resistin ⁶⁷; an ectopic fat deposition, mainly in liver and skeletal muscle ⁶⁸; and a mitochondrial dysfunction that will decrease insulin sensitivity, compromising β -cell function ⁶⁹.

1.3.3. Diabetes and immunity: Increased susceptibility to infections

Obesity is associated with low-grade inflammation and is probably contributing to the activation of immune system in patients suffering from T2DM ⁷⁰. The pathogenesis of T2DM is linked to both innate and adaptive immune responses that are recognized as important etiological components in the development of insulin resistance ⁷¹.

Innate responses in obesity/diabetes

An important accumulation of macrophages in the adipose tissue of obese mice models and humans was reported in previous studies. For instance, more than 50% of the adipose stromal cells in leptin receptor deficient obese ob/ob mice were identified as macrophages ⁷². Moreover, in obese people, an approximate of 40% of the adipose connective tissue cells was formed by macrophages ⁷³. The infiltration of macrophages has been correlated with adipocyte hypertrophy ⁷⁴, and also with the presence of necrotic adipocytes, suggesting that macrophages infiltration was due to the release of free lipid from necrotic cells ⁷⁵.

It is known that macrophages have a stunning plasticity so they can polarize from M1 classically activated type that produce high levels of ROS and NO but also TNF and IL-6 to combat infections, to M2 alternatively activated macrophages ⁷⁶, that are involved in collagen and polyamine production and healing functions as an answer to different stimuli ⁷⁷. Obesity adipose tissue is mostly populated by M1 macrophages with pro-inflammatory responses, and the numbers of M1 macrophages correlates with the presence of insulin resistance ⁷⁸. For example TNF has repeatedly been shown to inhibit different steps in the intracellular insulin signaling ⁷⁹. Evidence showed that when high fat diet-fed mice were changed to normal diet, macrophages polarized to M2 and insulin resistance was reduced ⁸⁰. Moreover, treatment of HFD-fed mice with pioglitazone (an insulin sensitising agent), resulted in reduction of M1 macrophages in tissue ⁷⁸. These studies show an important role of innate immune responses in insulin resistance, a hallmark in obesity and diabetes.

Glycation is a non-enzymatic reaction that occurs between a carbohydrate and a molecule with a free amino group, such as a protein ⁸¹. Glycation is a physiological and pathological process that produces the so-called glycated proteins and is entirely separate from glycosylation, which

represents an enzyme-controlled physiological process that occurs during synthesis of glycoproteins.

Chronic hyperglycemia in diabetic patients leads to glycation of immune components including immunoglobulins, diminishing their efficiency to bind complement⁸¹, NK cells, or suppressing inflammasome activation⁸² and therefore, increased susceptibility to infections. Particularly the accumulation of the reactive dicarbonyl metabolite methylglyoxal (MGO), is key in the development and accumulation of damage. In this line, MGO was found to inhibit T cell proliferative responses. Interestingly, this effect was reversed by N-acetyl cysteine (MGO scavenger)⁸³. Moreover, MGO inhibited the production of IL-10, IFN- γ and reduced the expression of MHC-I⁸⁴.

Adaptive responses in obesity/diabetes

Similar to macrophages, T lymphocyte accumulation has been found increased in obese adipose tissue⁸⁵. Moreover, in vitro studies showed that T cells inhibited the differentiation of pre-adipocytes to mature adipocytes, therefore contributing to hypertrophic adipocytes⁸⁶.

An accumulation of Th1 and Th17 cells was also found in obese adipose tissue, these cells were preceding macrophage infiltration. These proinflammatory T cells will promote macrophage polarisation towards M1 pro-inflammatory phenotype⁸⁷.

Lymphocyte deficient mice (such as *rag1*^{-/-} or *rag2*^{-/-} mice) are a very good model to explore the role of T cells. For instance, HFD-fed *rag1*^{-/-} mice showed increase fat mass and higher insulin resistance compared to HFD-fed lymphocyte-sufficient mice⁸⁸. Moreover, *rag1*^{-/-} mice that received different CD4⁺ T cell sub-populations indicated a reversal of insulin resistance only when they were transferred with Th2 differentiated cells, suggesting a protective role of Th2 cells from the development of insulin resistance in diet-induced obesity⁸⁸. In addition, depletion of Treg cells in leptin receptor deficient (db/db) mice lead to increased insulin resistance compared to Treg sufficient mice⁸⁹. On the other hand, hyperglycemia has been shown to lead to an impaired proliferative function of CD4 T lymphocytes⁹⁰.

Susceptibility to infections in DM

DM is a complex metabolic disease. The impairment or deregulation of immune responses of DM patients leads to their higher susceptibility to infections⁹¹. Epidemiological studies showed higher frequencies of infectious diseases in diabetic patients, including skin, genitourinary, gastrointestinal and respiratory infections⁹². For example, respiratory infections associated with DM are caused by *Streptococcus pneumoniae* and influenza virus⁹³. The prevalence of Hepatitis C has been reported to be increased in patients with DM⁹⁴.

Urinary tract infections (UTIs) are more prevalent in individuals with DM and may evolve to complications and/or serious manifestations⁹⁰. Acute pyelonephritis caused by *E. coli* or *Proteus sp.* is 4–5 times more common in individuals with DM⁹³. A study in a murine urinary tract infection (UTI) model showed that type 1 fimbriated uropathogenic *Escherichia coli*

(UPEC) had an increased adherence to the bladder in diabetic female mice compared with non-diabetic mice. Moreover, an increased concentration of two AGE's was found in superficial urothelial cells. Interestingly, this binding was inhibited by pre-treating diabetic mice with pyridoxamine (an AGE inhibitor) ⁹⁵.

Foot infections are a frequent cause of medical attention in DM ⁹⁶. An important causative agent of this infection is *Staphylococcus aureus*. A study performed with diabetic and insulin-responding non-obese diabetic mice (NOD) showed a higher expansion of skin lesions compared to non-diabetic controls. Remarkably, treatment with insulin ameliorated the skin infection produced by *S. aureus*. Moreover, in vitro studies showed that in the hyperglycemic blood the bacteria significantly multiplied, while the non-diabetic blood showed 68% death of bacteria ⁹⁷.

1.3.4. Tuberculosis

Tuberculosis (TB) is a bacterial infection produced by *Mycobacterium tuberculosis* (Mtb). It is transmissible from person to person, and it spreads through the air via bacteria containing droplets, mainly when an active infected person with Mtb in the lungs coughs, spits or sneezes ⁵.

An approximate of 10 million new cases of TB occurred in 2017. The incidence has been 1,5% approximately lower per year since 2000 (ref). TB is not only an important cause of morbidity and low quality of life, but it also caused the death of 1,57 million individuals in 2017. Of importance, the incidence of RR (rifampicin resistance) or MDR (multidrug resistance) was around 5,6% of total TB cases ⁹⁸, complicating TB control. Even though the incidence of TB decreased globally ⁹⁹, in resource-limited middle-income countries is still in the top five causes of death ¹⁰⁰, mainly because of poverty and poor access to health ¹⁰¹.

Co-morbidities with TB are increasing every year. Among all HIV infected, around 9% are also TB infected ⁹⁹. The risk of developing TB increases around three-times among DM patients ¹⁰². An improvement of nutrition and living conditions, helped to decline the prevalence of TB in the late 19th century.

Moreover, the introduction of Bacillus Calmette-Guerin (BCG) in 1920 helped to reduce the incidence of TB. BCG is the most widely used vaccine in the world. However, the efficacy of BCG is variable, and it has not solved the TB pandemic situation. BCG showed efficacy against childhood TB, but the effect in pulmonary TB in adults is still variable at most ¹⁰³.

The physiopathology of TB has been broadly studied, but some areas are still unexplored. Most individuals infected with *M. tuberculosis* remain chronically infected but are otherwise asymptomatic. However, 10% of infected individuals will develop active TB during their lifetime. Why some individuals are resistant to TB, but others show an array of pathologies is still not understood, but immune-bacterial interactions are of primary importance in controlling the development of infection.

Due to the mentioned mechanism of transmission, the primary target organ is the lung. Upon TB activation, the bacteria disseminates and disease can develop in any other organ resulting in extrapulmonary tuberculosis (EPT) ¹⁰⁴. Symptoms during TB are not very specific. TB presents a prolonged productive or dry cough, weight loss, fever, and night sweats ⁹⁹. The TB latency spectrum may be further subdivided into diverse stages associated to the related pattern of immune molecules expressed ¹⁰⁵

1.3.5. Mycobacterium tuberculosis

The causative agent of TB is an obligate intracellular pathogen whose principal hosts are human beings ¹⁰⁶. Mtb is an aerobic bacillus (motive for lung preference as habitat), but can also survive in hypoxic environments where it can switch its metabolism to a dormant or latent phase. Mtb is not-encapsulated, non-motile, acid-fast and it does not form spores ⁹⁸. The interactions between Mtb and the host are complex ⁹⁸.

Mycobacterium bovis - Bacillus Calmette Guerin (BCG)

Genomic differences between Mtb and BCG helped to define a particular region of difference called RD1, a main explanation of the lack of risk or progression to disease of around 4 million of children that have been vaccinated ^{107,108}.

The importance of RD1 is that it encodes a bacterial secretion system, known as ESX-1 ¹⁰⁹. ESX-1 delivers bacterial molecules into the host cell cytoplasm and allows bacterial to survive in such environment ¹¹⁰. The use of BCG in *in vitro* macrophage infections has been shown as a proper model to study inflammatory responses as well as host-mycobacterial interactions. Low multiplicity of BCG infection of macrophages has been shown efficient to stimulate high levels of bactericidal or bacteriostatic proinflammatory responses (i.e. IL-1 β and iNOS) and has a decreased cytolytic activity compared with Mtb infection ¹¹¹.

1.3.6. Immune responses to Mycobacterium tuberculosis

The host-pathogen interaction in TB is a complex with different responses played in response to Mycobacterium tuberculosis infection.

Innate responses to Mtb

To control the fragile balance between the host and the microbial infections, the host has developed a broad arsenal of innate immune mechanisms. These include physical barrier functions, soluble effectors such as complement and antimicrobial peptides, and phagocytic cells. The critical role of phagocytes in host defense lies in their rapid mobilization and recruitment into the tissue and their ability to recognize and inactivate pathogens following the activation of effector microbicidal responses. Phagocytes such as macrophages or neutrophils are recruited to the tissue in response to invading microorganisms. In the inflamed

tissue these phagocytes contribute to local inflammation. The inflammation encompasses an increased amount of monocyte extravasation and tissue accumulation.

The role and characteristics of innate immune responses during *M. tuberculosis* infection has been studied in diverse animal models. Murine, but also in rats, guinea pigs and non-human primates have been used for this purpose.

Following inhalation, Mtb move via the respiratory tract and until encountering alveolar macrophages, the most important cell target for this intracellular pathogen ^{112,113}. Macrophages internalize the bacteria by a receptor-mediated phagocytosis. Bacterial immune innate receptor agonists include peptidoglycans, glycolipids such as PIM, LAM and ManLAM and bacterial unmethylated CpG containing DNA. These molecules will activate Toll Like Receptors (TLR) and also other types of innate immune receptors such as NOD, C-type lectins (dectin-1 and 2), STING and mannose receptors ¹¹⁴. These receptors are single membrane-spanning receptors that will recognize conserved molecules derived from microorganisms ¹¹⁵. Human studies showed variable TB susceptibility in patients with TLRs polymorphisms ¹¹⁶. The interaction between Mtb ligands and TLRs is mediated by the Myeloid Differentiation Primary Response 88 (MyD88), that will later cluster with the IL-1R associated kinase (IRAK4). The complex will activate TNF receptor associated factor (TRAF6), finally resulting in the translocation of the NF- κ B transcription factor into the nucleus and the transcription of inflammatory mediators ¹¹⁷. TLRs also activate mitogen-activated protein kinases (MAPK) that activates other pro-inflammatory transcription factors (AP-1).

So far, it is known that TLR2 and TLR9 play a minor role in TB infection if any ¹¹⁸. In vivo studies showed that TLR4-null mice resistance to Mtb infection is slightly decreased, together with an increase of bacterial growth and diminished survival. On this line, macrophages from TLR4-deficient mice produced less TNF- α upon stimulation with Mtb than controls ¹¹⁹. In vivo, TLR2-deficiency also lead to diminished resistance against a higher dose of Mtb ¹¹⁷, but more studies are needed to keep exploring the function of TLR2 and TLR4 and maybe other TLRs involved in TB infection.

TLR as well as signaling via other receptors results in release of reactive oxygen and nitrogen species and also the secretion of chemokines and cytokines. The later will mediate the and recruitment and activation of PMNs, macrophages and dendritic cells ¹²⁰.

The bactericidal/bacteriostatic arsenal of murine macrophages above mentioned has different degrees of efficiency against *M. tuberculosis* ¹²¹. Among mycobactericidal mechanisms of macrophages a relevant one is the de novo expression of the inducible nitric oxide synthase (iNOS), which results in the production of the reactive nitric oxide (NO) by the conversion of arginine into citrulline ¹²². The expression of *inos* mRNA occurs directly in response to mycobacterial innate immune stimulation, and occurs in synergy by TNF (also secreted by macrophages after innate immune activation) and IFN- γ released by CD4+T cells ¹²³.

The relevance of iNOS derived NO in the microbicidal activity in human macrophages is not completely understood. 1,25-dihydroxy vitamin D₃ (1,25-(OH)₂D₃) in combination with

IFN- γ and TNF- α activated macrophages inhibited grow and/or killed Mtb in human macrophages^{124, 125}. 1,25-(OH)₂vitamin D₃ induced the expression of NOS2 inhibiting Mtb activity in human HL-60 macrophage-like cell line¹²⁶.

Adaptive responses to Mtb

The role of T cells to protect the host against Mtb infection is crucial. Activation of both CD4⁺ and CD8⁺ T cells is observed in active TB in humans, as well as in infected mice¹²⁷. CD4⁺ Th1 cells are thought to be the most critical for bacterial control¹²⁷. CD4⁺ T cells recruited to the infected lung are thought to control infection by producing IFN- γ in response to mycobacterial antigens presented by macrophages^{127, 128, 129}. In turn, IFN- γ will activate macrophages to kill the intracellular Mtb through NO and reactive oxygen intermediates¹³⁰, and by inducing the formation of phagolysosomes^{131,122}.

Mice lacking CD4 T cells, IFN- γ , IL-12 signaling (a pathway required for Th1 development), or T-bet (a transcription factor requisite for Th1s) are very susceptible to infection with *M. tuberculosis*¹²⁷. Alike, humans with genetic deficiencies in IFN- γ or IL-12 signaling¹³², as well as HIV-infected individuals depleted of CD4 T cells¹³³, are severely restricted in their ability to contain mycobacterial infections, including TB. CD8 T cells can also help control Mtb by both perforin-mediated cytolysis of infected macrophages and direct killing of *M. tuberculosis*¹³⁴.

However, and, different from other infections, T cells will take longer time to be activated in the draining lymph nodes and migrate to the lungs. For instance, it has been shown that in infected animals specific lung T cells will recognize bacteria in approximately 3 weeks after infection, compared to 5-7 days occurring in other infections^{135,127}. *M. tuberculosis* may directly infect dendritic cells, and impair their migration to the draining lymph nodes and thus the activation of naïve T cells will be delayed¹³⁶. At a later stage, when T cell priming is initiated in the draining lymph nodes, the inflammatory milieu of Mtb infection promotes expansion of a highly suppressive population of Mtb-specific regulatory T cells (Tregs). The Tregs will impair the activation of anti-mycobacterial T cells, to prevent autoimmunity¹³⁷. Regulatory T cells were found increased in TB infected lungs from mice¹³⁸ and humans with active TB¹³⁹. Studies in mice lacking Treg subsets were performed. However the findings were a bit controversial, as Treg depleted mice indeed presented less bacterial load, but the mice developed autoimmune damage¹³⁸. The proper activation of T cells will also be disturbed by the presence of regulatory cytokines (IL-10 and TGF- β) and other immunosuppressive factors (i.e. lipids) and by the high bacterial burden¹³⁶. All of these results in the presence of T cells required to control the infection but with a limited ability to clear it.

Granuloma formation

A human granuloma can be defined as a compact and organized aggregate of mature macrophages that develops as a tissue reaction in response to a persistent stimulus ^{140,141}.

Usually macrophages take the central part and they can present additional changes, like bigger and mature with ruffled cell membranes, known as epithelioid cells. They can also fuse into multinucleated giant cells or differentiate into foamy cells with lipid accumulation ¹⁴². Granulomas may present central region of necrosis due, a “caseum” and are typical in TB pathology ¹⁴³. Moreover, this formation seems to be helpful to contain the bacteria, but a rupture of the structure can lead to disseminate the infection ¹⁴⁴.

The granuloma is populated by other cell types apart from macrophages like neutrophils, dendritic cells, NK cells, fibroblasts and B and T cells. Moreover, epithelial cells also participate in the formation of granuloma and are the ones surrounding the structure ¹⁴⁵

A co-evolutionary perspective seems to be accurate to explain the function of a granuloma, since it is not only a structure that will limit bacillary growth and the niche where infection is sequestered, but also will be a successful part of Mtb life cycle, helping in its transmission and propagation ¹⁴⁰.

1.3.7. Tuberculosis and diabetes comorbidity (TB-DM)

The interaction between diabetes and tuberculosis is an imperative and complex global health concern ¹⁰². Diabetic patients have a three-four fold increase risk to develop TB compared to non-diabetic persons ¹⁴⁶.

DM cases are rising not only in high but also low-income countries, leading to “double-trouble” effect ¹⁴⁷. DM might increase the severity of TB disease, and the relapse and treatment responses ¹⁴⁸.

The occurrence of TB-DM has been noticed several years ago. Already in 1934 studies in patients showed that TB in adults and adolescents with DM was more common than expected, but no specific clinical issues were found when compared to non-diabetic patients ¹⁴⁹. Recently, several epidemiological studies supported that both the TB prevalence and the risk of infection increased among DM patients. This created an interest in understanding the involved mechanisms of comorbidity ^{146,150}.

Regarding the clinical presentation and severity of infection in TB-DM patients, a lower lung involvement in radiographic findings in TB-DM patients was more pronounced compared to non-diabetic ¹⁵¹. Moreover, a multilobar-cavity pattern was more usual in TB-DM patients ¹⁵².

It has been mentioned before that DM alters protective immunity to infections. Some studies indicated that mycobacterial burden might be higher in DM than controls, which could also show an impaired response to treatment ¹⁴⁶. In one study, similar sputum conversion proportions were found after two months of TB treatment in both TB-DM and non-diabetic TB

patients. In contrast, another study showed a longer conversion time of sputum in DM patients^{153,154}. Alterations in the immune responses were evident in one study on TB-DM patients, showing that innate and Th1 responses were significantly higher compared to controls¹⁵⁵. The effect was consistently and significantly more marked in DM patients with chronic hyperglycemia.

The possible mechanisms involved on the increased susceptibility to Mtb infection were studied in few DM animal models. DM is modelled in both obese and non-obese animal models with varying degrees of insulin resistance and beta cell failure.

In chemically, streptozotocin-induced mice models of DM, a high percentage of the endogenous β -cells are destroyed, and thus, there is little endogenous insulin production, leading to hyperglycaemia and weight loss. A disadvantage with chemically inducing DM is that the chemicals can be toxic at other organs of the body. Mtb-infected streptozotocin-induced DM mice showed ten times higher phagocytic activity of macrophages and also higher mortality than normal mice¹⁵⁶. Moreover, streptozotocin-induced diabetic mice had higher bacterial load than normal mice (own data, unpublished).

Monogenic models of obesity are commonly used in type 2 DM research. The most widely used monogenic models of obesity are defective in leptin signalling. Leptin stimulates satiety, and thus, lack of leptin in these animals causes hyperphagia and obesity. These models include the leptin deficient ob/ob, the leptin receptor deficient db/db mouse and the leptin receptor deficient Zucker Diabetic Fatty rat^{136, 157}. Obese ob/ob mice showed hyperglycemia and lack insulin production (ob/ob) displayed higher bacterial loads in the lungs at different time points of Mtb infection¹⁵⁸. Mtb-infected db/db mice showed disorganized granulomas, neutrophilia and reduced B cell migration to the lungs. Db/db mice also showed increased bacterial load¹⁵⁹. Despite that some lights were shown regarding TB-DM synergism, the mechanisms behind this interaction remains incompletely understood.

1.3.8. Hypoxia inducible factor (HIF-1)

The cells of most living organisms require oxygen to produce energy as ATP and fulfil their metabolic activities, development and growth. Hypoxia can result from a failure in the delivery of oxygen to cells. Failures might include decreased pressures of oxygen (i.e. at high altitude), problems with diffusion of oxygen in the lungs, insufficient accessible hemoglobin, problems with blood flow to the tissue, and problems with breathing tempo^{160,161}. Oxygen deprivation in different tissues generate hypoxic environments that may cause cell damage, morphological and functional changes. Hypoxia can cause circulatory disorders, and heart, lungs and brain necrosis. It might also diminish responses to tumors. To prevent this, mammals have developed a sophisticated physiological network to maintain oxygen homeostasis. One of the major characteristics of this network is the ability to sense and respond to low-oxygen conditions. The initiation of these responses to hypoxia is quite fast and involves both transcriptional and post-transcriptional mechanisms¹⁶².

Although hypoxia is often thought of as being a pathological phenomenon, the mammalian embryo in fact develops in a low-oxygen environment and hypoxia affects ultimately morphogenesis of the embryo and placenta ^{163,164}.

HIF-1 was described due to its role in inducing erythropoietin (a hematopoietic growth hormone) in renal interstitial fibroblasts under hypoxia. as this will increase blood oxygen transport ¹⁶⁵. HIF-1 is a key regulator of a broad range of cellular responses to hypoxia in mammalian cells. There are over 200 genes that increase their gene expression either directly or indirectly by HIF-1 ¹⁶⁶ of which 70 have been reported to be directly induced by HIF-1 ¹⁶⁷. Some target genes promote anaerobic metabolism to reduce oxygen use, while others “alleviate” hypoxia by acting non-cell-autonomously to extend and modify the surrounding vasculature ¹⁶³. HIF pathways are involved in erythropoiesis, angiogenesis, cell growth and differentiation, survival and apoptosis; all are critical factors in development, physiology and disease ¹⁶⁸.

HIF-1 is a heterodimeric helix-loop-helix transcription factor formed by two subunit proteins. The expression and stability of the α subunit (HIF-1 α) is regulated by a family of oxygen- and iron-dependent prolyl hydroxylases. The subunit (HIF-1 α) is constitutively expressed and is also called ARNT (aryl hydrocarbon receptor nuclear translocator). In normoxic conditions HIF-1 α is hydroxylated on proline residues. The hydroxylation targets HIF-1 α for degradation in a process dependent upon the recognition by the von Hippel–Lindau tumour-suppressor protein (vHL) ¹⁶⁹. VHL is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1 α for ubiquitylation and proteasomal degradation ¹⁷⁰.

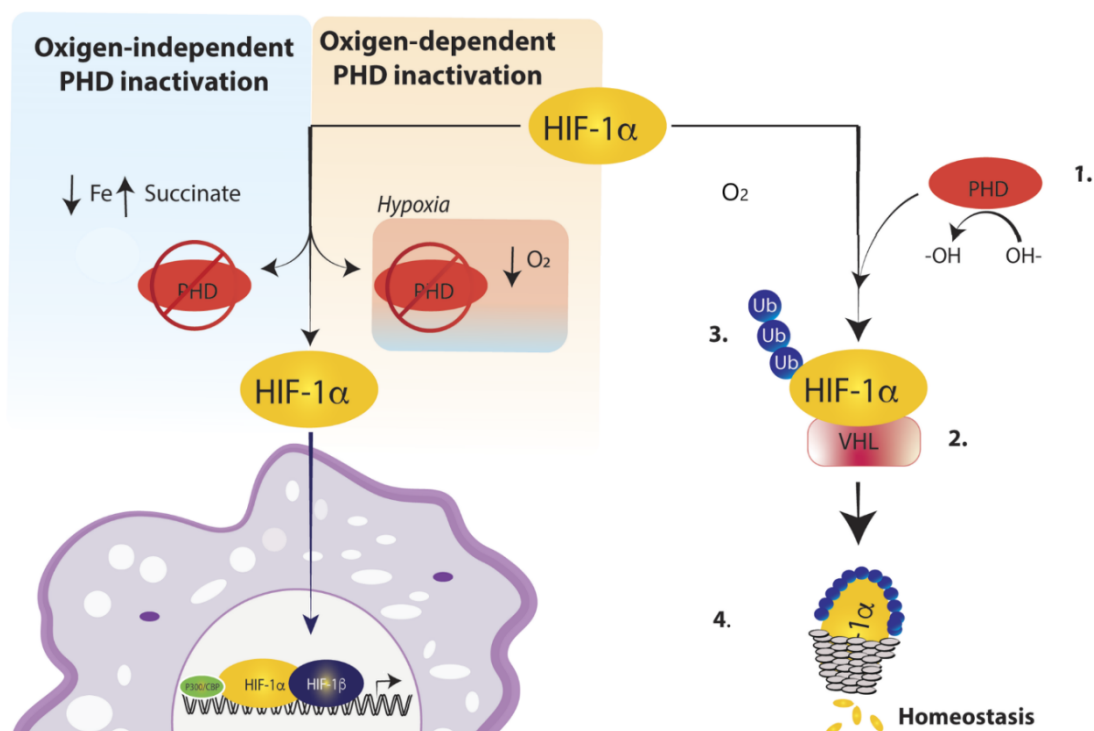


Figure 2. HIF-1 α stabilization pathways in an Oxygen-dependent and Oxygen-independent manner. HIF-1 α degradation: 1. Hydroxylation; 2. Recognition; 3. Ubiquitination 4. Proteasomal degradation.

If the prolyl hydroxylases are inhibited, HIF-1 α will not be degraded, but accumulated. HIF-1 α will then translocate to the nucleus and dimerize with HIF-1 β . The heterodimeric complex HIF1 will then bind to the HRE (Hypoxic reactive elements) in promoter or enhancer sequences of target genes. A nuclear activator and a corepressor regulate HIF-1 transcriptional activity. The coactivators are p300 and CBP, and they will catalyse acetylation of histone proteins and initiation of gene transcription. The corepressor has multiple isoforms and that are regulated by histone deacetylases (HDACs) ^{168, 169, 171}. A simplified scheme of HIF-1 α stabilization in hypoxic or normoxic conditions is depicted in Figure 2.

1.3.9. HIF-1 α and immunity/infections

Innate immunity and HIF-1 α

During infection, the intense metabolic activity of activated phagocytes will, together with the oedema, vascular damage and tissue destruction, reduce the amount of oxygen in the tissue. The inflamed tissue is characterized by low levels of oxygen and glucose coexisting with high concentrations of free oxygen radicals and lactate ¹⁷². Phagocyte activation in these conditions generates ATP via glycolysis.

HIF-1 α is expressed in most if not all innate and adaptive immune populations including macrophages ¹⁷³, neutrophils ¹⁷⁴, dendritic cells ¹⁷⁵, and lymphocytes ¹⁷⁶.

The control of cellular metabolism by HIF-1 α has been shown to play an important role in cells that are protagonists of the first line of defence against infections. While the development/differentiation of innate cells like monocytes, macrophages and neutrophils doesn't seem to be affected by HIF-1 α deletion¹⁷³, the activation of cells of the innate immune system by microbial products or infection switch their metabolism from mitochondrial oxidative phosphorylation to HIF-1 α -mediated aerobic glycolysis (also known as the Warburg effect in cancer cells), as well as by an upregulation of pathways involving oxidative defense responses, arginine metabolism, and synthesis of bioactive lipids ⁶³. This will provide an adaptation to demanding conditions such as lower levels of oxygen in injured tissues in order to maintain viability and activity. Because the upregulation of most glycolytic pathway enzymes is mediated by HIF-1 α ^{177 169}, a role for this transcription factor in supporting phagocyte function during inflammation could be predicted.

Macrophages with impaired HIF-1 α levels were less capable to aggregation, invasion and motility¹⁷⁸. HIF-1 will boost microbicidal activation of phagocytes. HIF-1 will promote the activation of the innate immune cells, promoting phagocytosis, increasing phagocyte lifespan by inhibiting apoptosis, releasing pro-inflammatory cytokines and antimicrobial peptides, and importantly, activating production of NO¹⁶⁹ as well as of reactive oxygen species. Of importance phagocyte activation, was shown to be dependent on HIF-1 α even when it occurred in normoxic conditions ¹⁷⁹.

It is important to mention that every inflamed tissue will activate the transcription factor NF- κ B. Moreover, the hypoxic environment created by inflamed / infected tissues has been shown

to activate both NF- κ B and HIF1 (references). Therefore, an important crosstalk between NF- κ B and HIF-1 α was investigated. In fact, there are evidences that NF- κ B will modulate and stabilize HIF-1 after microbial stimulation (references). However, the situation of NF- κ B when HIF-1 is first stabilized is still not completely unravelled. For instance, HIF-1 α has been shown to restrict NF- κ B transcriptional activity *in vivo* and *in vitro* under inflammatory conditions ¹⁸⁰, but this mechanism needs to be studied further.

Cytokines and HIF-1

IL-1 β is a major macrophage-derived pro-inflammatory cytokine that is upregulated in both inflammation and infection. The maturation of IL-1 β is mainly due to activation of the inflammasome, a complex of NLRP-3, an adaptor protein and caspase-1 that will be activated in response of microbial products among others ¹⁸¹. A study about HIF-1 and IL-1 β in an ovarian carcinoma cell line, showed that IL-1 β induced the expression of adrenomedullin mRNA (a peptide related with vascular homeostasis in hypoxic environments) by activating HIF-1 under normoxic conditions, and presented increased accumulation and nuclear translocation of HIF-1 α ¹⁸². Recently, an interesting link between succinate accumulation in LPS stimulated macrophages and IL-1 β has been described. Succinate is transported from the mitochondria via the dicarboxylic acid transporter to the cytosol, impairing PHD activity and therefore promoting HIF-1 α stabilization (pseudohypoxia), this effect produced an important augmentation of *il1b* mRNA of BMM treated with diethyl succinate and stimulated with LPS. In contrast, BMMs from HIF-1 α deficient mice had less IL-1 β protein and mRNA levels after stimulation, and treatment with diethylsuccinate had no effect on IL-1 β secretion.

In line with this, PKM2 (pyruvate kinase M2), an inactive dimer of pyruvate kinase enzyme, has been shown to be a critical modulator of IL-1 β production through HIF-1 stabilization, as well as of glycolytic reprogramming in LPS-activated macrophages. There is a clear inhibition of LPS-induced HIF-1 α and IL-1 β as well as the expression of important HIF-1 α dependent genes when PKM2 is activated as a tetramer, therefore this can be an important target for improving immune responses ¹⁸³.

Several studies have correlated and increased production of NO with HIF-1 α stabilization in infections. For instance, it has been shown that HIF-1 is stabilized with deferoxamine (DFO). DFO induced *inos* mRNA expression and iNOS transcription in IFN γ -treated murine macrophages (reference). DFO induced the binding of HIF-1 to a hypoxia-responsive enhancer in the *inos*- gene promoter ¹⁸⁴. Moreover, a direct transcriptional activation of *inos* gene expression in myocardial cells exposed to hypoxic conditions has been shown ¹⁸⁵. In addition, the accumulation of HIF-1 α was prevented by inhibition of NOS activity, indicating that NO might also be responsible for the accumulation of HIF-1 α ¹⁸⁶. HIF-1 α function requires NO production, and that HIF-1 α and iNOS are linked by a positive feedback loop that amplifies macrophage activation ¹⁸⁷.

Adaptive immunity and HIF-1 α

The consequences of HIF-1 α functions are not only limited to the myeloid lineage. There is considerable evidence that HIF-1 α has an important role in the modulation of T cell functions. As with innate immune cells HIF-1 functions in T cells can be increased in an oxygen-independent manner, via T cell receptor activation or by defined cytokines.

Studies on HIF-1 α function in CD8⁺ T cells infiltrating T cells in tumours showed that T cells utilized HIF-1 to adapt to the hypoxic tumour microenvironment (ref). HIF-1 α , but not HIF-2 α was essential for establishing effector functions of CD8⁺ T cells and loss of HIF-1 α in CD8⁺ T cells reduced tumor infiltration and tumor cell killing ¹⁸⁸.

Another study used mice with VHL deletion in CD8⁺ T cells, leading to HIF-1 α stabilization. This deletion promoted the glycolytic metabolism after the T cell receptor activation. Such HIF-1 α activation of glycolysis accelerated the differentiation of CD8⁺ memory T cells during viral infection. Increased glycolysis was required for the posterior formation of long-lived effector-memory CD8⁺ T cells, which showed a mitochondrial oxidative phosphorylation metabolism. These results indicated an important link between cellular metabolism and memory CD8⁺ T cell differentiation¹⁸⁹. Yet, the role of HIF-1 in the regulation of adaptive immunity is still controversial and needs further investigation.

Some studies have shown that HIF-1 α instead might suppress some effector functions of CD4⁺ T cells. In fact, the release of TNF and IFN- γ was increased in T cells lacking HIF-1 α gene compared to wild type T cells ^{190 191}. Thus, while metabolic reprogramming occurs during the activation of all T cells their effects can be different with respect to the usage of HIF-1 pathway.

1.3.10. HIF-1 role in obesity/diabetes

Obesity, as discussed above, is a health problem that frequently co-exists with type 2 DM, leading to the so called "diabesity epidemic" ¹⁹². The rising incidence of type 2 DM among children and adults is related to the epidemic of obesity. The adipose tissue has been shown to create an hypoxic environment caused by the presence of a moderate chronic inflammation ¹⁹³. The obesity-associated factors hypoxia, adipogenesis and insulin, all elevated HIF-1 α protein, but only adipogenesis and insulin enhanced HIF-1 α mRNA activity. Moreover, VEGF (a major target gene of HIF-1) was also upregulated due to the need of for angiogenesis for further growth of the adipose tissue ¹⁹⁴.

HIF-1 and Hyperglycemia

The regulation of hyperglycemia on HIF-1 α activity is still not completely understood. On one side hypoxic lesions from different organs like heart and retinas in diabetic rats showed HIF-1 upregulation ¹⁹⁵. On the other hand, there are few hypotheses explaining the HIF-1 α deregulation by hyperglycemia. For instance, a negative effect of reactive oxygen species (ROS) through methylglyoxal (MGO) has been postulated. MGO is a dicarbonyl compound that can be found in all cells since it is a by-product of cellular metabolism, more specifically of

glycolysis¹⁹⁶. This means that if the concentrations of their precursors are elevated, as for example during hyperglycemia MGO levels will increase. MGO are highly reactive glucose metabolites that form stable adducts (glycation) primarily with arginine residues of intracellular proteins¹⁹⁷. Among these proteins, MGO can cause a covalent modification of HIF-1 α and a decreased dimerization of HIF-1 α and HIF-1 β , reducing the binding of HIF-1 to the HRE (hypoxia responsive elements). The covalent modification of p300 will lead to inhibition of the interaction of CTAD (carboxy-terminal transactivation domain) and p300, which will decrease the transactivation ability of HIF-1 α ¹⁹⁸. Moreover, the covalent modification leads to HIF-1 α proteasomal degradation¹⁹⁹. Figure 3 describes the possible pathways of HIF-1 α destabilization through methylglyoxal (MGO).

MGO is a potent inducer of oxidative stress and AGEs (advanced glycation end products). In diabetic patients both MGO and oxidative stress levels are increased, and accompanied by a reduction of antioxidant defences and a constant ROS/AGE formation²⁰⁰.

A direct regulatory role of ROS on HIF-1 α has received attention. However, the existing body of literature shows contradictory findings²⁰¹. A reaction between O₂ and NO results in a decreased NO concentration, reducing NO-induced HIF-1 α accumulation and activation²⁰². On the other hand, HIF-1 α accumulation in hypoxic tissues is blocked by antioxidants²⁰³.

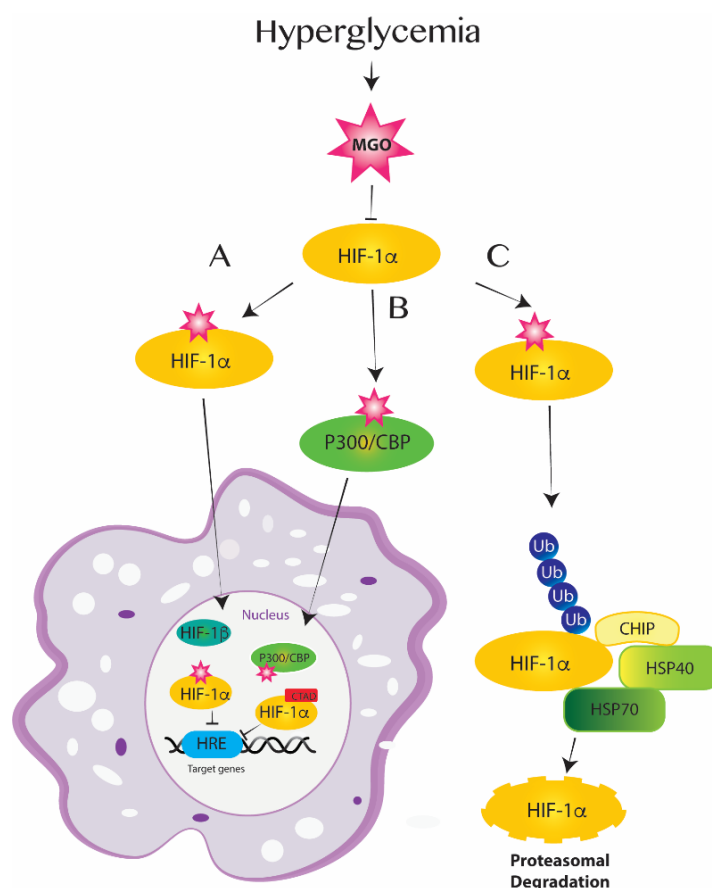


Figure 3. Possible mechanisms of Hyperglycemia and HIF-1 α destabilization through Methylglyoxal (MGO). **A.** MGO causes a covalent modification of HIF-1 α and decreases its dimerization with HIF-1 β and binding to HRE; **B.** A covalent modification of p300 inhibits its interaction with CTAD, therefore decreasing the transactivation ability of HIF-1 α and **C.** A covalent modification of HIF-1 α increases its association with HSP40/70, which recruits CHIP and produces HIF-1 α proteasomal degradation. Modified from Xiao et al

Finally, it is important to keep in mind that in DM both hypoxia and hyperglycemia are present. Thus, cells might not be capable to respond well to hypoxia, increasing the risk of complications. During DM the expression of HIF-1 α has been shown to be reduced in a relatively major area of hypoxic lesions ¹⁹⁵. While hyperglycemia has been suggested to modulate HIF-1 α functions, whether this malfunctioning of the HIF-1 α pathway can underline the failure to control infections in hyperglycemic conditions has so far not been studied.

1.3.11. HIF-1 role in Tuberculosis

Among other pathological features in TB, the existence of hypoxic lesions has been demonstrated. Direct determination oxygen tension in rabbits ²⁰⁴, and indirect measurements in macaques and humans using hypoxia-sensitive probes demonstrate that several TB lesions *in vivo* are hypoxic ^{205 206}. In man, TB granulomas can consist of a caseous necrotic centre surrounded by a cuff of T cells. Mycobacteria often reside within necrotic tissue that has no obvious supply of oxygen ²⁰⁴ were it differentiates into a dormant/ latent stage. It is likely thus that HIF-1 α plays thus a role in the immune control of bacteria in the lesions.

In vitro and murine studies have shown an upregulation of HIF-1 α during Mtb infection, opening doors to study a possible role of HIF-1 α in regulating immune protection ^{187, 207}. The expression of HIF-1 α showed a progressive increase during Mtb infection. HIF-1 α expressing macrophages were already detected in the first month of infection in mice. The HIF-1 α expression was higher during the late phase (day 60) ²⁰⁸. A strong expression of HIF-1 α was observed in TB granulomas ^{209, 208}.

Mice lacking HIF-1 α in the myeloid lineage were highly susceptible to Mtb infection and exhibited defective production of inflammatory cytokines and microbicidal effectors. In one of these studies HIF-1 α was shown to regulate the responses to IFN- γ . On the other hand, HIF-1 α levels were higher in IFN- γ activated, *Mtb*- infected macrophages. Although IFN- γ is a critical host-protective cytokine against intracellular pathogens, HIF-1-deficient macrophages permitted *M. tuberculosis* growth even after activation with IFN- γ ²⁰⁹. RNA-sequencing demonstrated that HIF-1 α regulated half of the IFN- γ inducible genes in Mtb infected macrophages ²¹⁰. HIF-1 α was found to be linked to iNOS and NO by a positive feedback loop that amplifies macrophage activation during Mtb infection. Moreover, NO at the same time as shown to inhibit NF- κ B activity to prevent hyperinflammatory responses, regulating thus on one side the HIF-1 α -mediated microbicidal response and on the other limiting the NF- κ B inflammatory damage ¹⁸⁷.

Lipid droplets are formed in *M. tuberculosis* infected macrophages. It has been shown that IFN- γ driven, HIF-1 α dependent signaling pathway, redistributes macrophage lipids into lipid droplets. *M. tuberculosis* were able to acquire host lipids in the absence, but not in the presence of LDs. HIF-1 α and IFN- γ were also required for the formation of lipid droplets in the lungs of *M. tuberculosis* infected mice ²¹¹.

Studies performed in zebrafish also showed an important participation of HIF-1 α in a *Mycobacterium marinum* infection (*Mm*) ^{212, 213}. *Mm*-infected zebrafish mimics some key features of human TB, including formation of granulomas. When HIF-1 α was stabilized in zebrafishes the bacterial burden was decreased. This effect was mediated by a NO-dependent mechanism ²¹². An upregulation of IL-1 β transcription was detected in the forming stage of the granuloma of zebrafishes, at 6 to 8 h post-infection. This correlated with an early upregulation of HIF-1 α signalling showing an immediate proinflammatory response. Of note, HIF-1 α activation was transient and not sufficient to control the infection, but when HIF-1 α was primed

with high IL-1 β and NO a lower infection levels were achieved ²¹³. In a model of mouse infection with *Mycobacterium avium* that generates encapsulated necrotic granulomas, the presence of necrosis was accelerated in the absence of HIF-1 α ²¹⁴.

Studies about the role of HIF-1 α in human TB are still scarce. Granulomas with a necrotic core in the lungs of TB infected patients showed hypoxic areas. In this line, high HIF-1 α expression was detected by immunohistochemistry in post-mortem lung samples from TB patients compared to controls ²⁰⁵. Moreover, a positive correlation between HIF-1 α nuclear expression and the presence of Mtb antigens was found, as well as a higher expression of both compared to lung tissue from the control group ²⁰⁵.

2 AIMS

The general aim of my thesis was to study the interactions between nutrition, immune responses and infections. This aim includes three important areas that are continuously interacting in health and disease and are quite broad. Very different and specific set ups were used to address these interactions in the studies herein presented.

- The first aim (related to paper I) was to study and compare the nutritional status in children from different socio-demographic areas, opting for communities situated in the highlands (3600-4000 masl) and lowlands (600 to 800 masl) from Bolivia. In line with this, another purpose was to study the prevalence of intestinal parasitic infections (IPIs) in these children and to find a possible correlation between nutritional parameters and IPIs prevalence. This study permitted to explore and understand the interaction between nutritional and environmental conditions in children.
- The second aim (related to paper II) was to investigate mechanisms that can explain how intestinal nematode infections modulated immune responses to secondary infection or vaccination at distal sites. This study permitted to investigate the outcome of chronic worm infections and the modulation of immune responses.
- The third aim (related to paper III) was to investigate the effect of obesity/diabetes in the outcome of tuberculosis infection and the role of HIF-1 α in this co-morbidity. In this study, the molecular mechanism behind the detrimental interaction between DM, a metabolic disorder associated to obesity, and tuberculosis was studied. A possible impairment of the function of HIF-1 α , a transcription factor involved in the modulation of metabolic and immune protective responses against infections, in the TB-DM comorbidity, was investigated.

3 METHODS

The procedures and techniques used to perform all the studies that comprise this thesis are described in every paper/manuscript. In this section, the intention is to expand the information about the experimental mice models and in vitro methods used to investigate and reach the aims.

3.1 EXPERIMENTAL TUBERCULOSIS: MICE MODELS OF TB

In paper III a model of tuberculosis that offers efficient tools to analyse the host response in Mtb infection was used. Most of the studies to understand host-pathogen interaction were performed in mice. Here we describe the benefits and weaknesses of this model ^{215, 216}.

Advantages and Disadvantages of a TB mice model

ADVANTAGES	DISADVANTAGES
<ul style="list-style-type: none">• Study the molecular basis of Mtb virulence• Availability (resistant/susceptible strains)• Targeted mutant strains (could develop similar human pathology)• More analytical reagents available• Less expensive than host species• Similar immune responses	<ul style="list-style-type: none">• Disease pathology differs from human TB• It lacks a latency stage• Still controversial if it is an appropriate model to answer some of the problems in TB human disease: susceptibility, major lung involvement, vaccination difficulties

We used a resistant strain (C57BL/6) with a median survival time of 250 days when infected via aerosol with a low dose Mtb Harlingen strain. Susceptible strains, like DBA/2, C3H, CBA and others have a median survival time of 100 days ²¹⁷. Db/db mice were also infected.

In paper III, 100% of mice used in all the experiments were infected via aerosol. The dose used was ca 200 CFU (colony forming units) when measured in the lungs one day after infection. Previous set up in our lab showed that mice infected via aerosol with the same technique presented consistent numbers of mycobacteria implanted in the lungs ²¹⁸.

A nose-only aerosol exposure unit (In-tox Products, New Mexico, USA) was used for infection. A 15-ml suspension of 1×10^6 Mtb per ml was loaded into a nebulizer, and placed animals inhaled the bacteria aerosol within 20 min. The advantages of an aerosol infection compared to an intravenous route are several. For instance, mice inoculated intravenously develop a systemic infection that is distributed to several organs differing on the priority of infection; generally, around 90% of the bacilli localizes in the liver, 10% in the spleen and around 1% in the lungs. Therefore, lung infection iv route is possible, but the CFU load will be lower, around 5×10^5 CFU in the lungs ¹²⁹.

The aerosol infection simulates the route used in the human natural infection with Mtb which targets the lung as a primary organ. Mtb has a very slow replication. Importantly, about 20 days after infection, an initial period of logarithmic bacterial growth takes place in the lungs, followed by a stationary phase of infection that is dependent on the onset and maintenance of adaptive immunity. This period will persist for 250-300 days ²¹⁹. We observed the presence of granulomas in all infected mice. Lungs showed several scattered macroscopic granulomas combined with disseminated inflammatory lesions. The severity of these lesions apparently increased somewhat from one to three months of infection.

The infection will not be confined only to the lungs, but, as in the human infection, dissemination via lymph and blood will take place, infecting other organs. The bacterial level in these organs will be lower than in the lungs ²²⁰.

In our experiments, mice were sacrificed either after one or three months of infection. Bacteria from lung and spleen lysates were plated and quantified on Middlebrook 7H11 agar containing 10% enrichment of oleic acid, albumin, dextrose, catalase, 5µg amphotericin B per ml and 8 µg/ ml polymyxin B grown for 3 weeks at 37°C.

3.2. THE DB/DB MODEL

A very well defined obese/diabetic model was used to study the outcome of Tuberculosis infection in paper III. This is a mutant congenic strain known as the BKS-Lepr^{db/db}/JOrlRj and lacks the functional leptin receptor ²²¹. Some important characteristics of the model will be explained in this section.

General description of the db/db model ^{221,222}

MICE STRAIN	MODIFICATION	EFFECT
BKS-Lepr ^{db/db} /JOrlRj Background (C57/BL/6J)	Mutation of the Lepr ^{db} gene located in chromosome 4	Compensatory hyperplasia of the β cells in the islets of Langerhans
MODEL	PHENOTYPE	SYMPTOMS
Type II Diabetes (obesity-related) <ul style="list-style-type: none"> • Hyperglycemic • Hyperinsulinemic • Insulin resistant • Peripheral neuropathy, Cardiomyopathy • Lipocyte-defects • Hypothalamus, pituitary gland and pancreas alterations 	Black, obese at 3 to 4 weeks of age <ul style="list-style-type: none"> • Insulin increase in plasma at days 10 to 14 • Hyperglycemic at 4 to 8 weeks • Increased circulating total cholesterol (including HDL, LDL and VLDL) 	Affected mice present: <ul style="list-style-type: none"> • Excessive hunger, increased appetite (Polyphagia) • Excessive thirst (Polydipsia) • Frequent urination (Polyuria) • Delayed wound healing

Six-week old BKS(D)-Lepr^{db/db}/JOrlRj (db/db) leptin receptor deficient mice and C57BL/6 mice were used. The mice are already obese at this age. Weight was measured before and throughout the infection and compared to that of WT C57BL/6 animals. Blood measurements like glucose, HbA1c (glycated haemoglobin), cholesterol and triglycerides were determined periodically. Weekly measurements of weight and blood glucose were performed after infection. Cholesterol and triglycerides were determined every two weeks and HbA1c was measured before the challenge and after three months of infection.

Most of the mice presented polyuria, polyphagia and polydipsia; therefore, constant access to food and water and cage changes were performed. No deaths were recorded during the infection but db/db mice lost weight at starting before 3 months of infection.

3.3. HIGH GLUCOSE EXPOSURE OF MURINE BONE MARROW DERIVED MACROPHAGES (BMM)

In paper III, all *in vitro* experiments were performed on murine BMM. Tibias and femurs from mice of the strain of interest were harvested and the bone marrow was flushed with a PBS-filled syringe. Bone marrow cells differentiate into BMM 6 days after culturing with M-CSF containing medium in macrophages as described ²²³. In all the experiments, BM cells were exposed to normal “euglycemic” 5mM glucose conditions using DMEM media throughout. Alternatively, DMEM-25 mM glucose media was replaced 72 h after BM culture. Next, BMM were infected and further cultivated in high or normal glucose containing media.

4 RESULTS AND DISCUSSION

4.1 PAPER I: DIFFERENCES IN NUTRITIONAL AND HEALTH STATUS IN SCHOOL CHILDREN FROM THE HIGHLANDS AND LOWLANDS OF BOLIVIA

Malnutrition is a global health concern that affects the development of every individual and the collective population. Bolivia is a country that has one of the highest prevalence of undernutrition among the Andean countries and is still one of the poorest in Latin América. However, a map showing the nutritional prevalence in different regions of Bolivia is still missing. Data regarding the nutritional situation in remote communities of Bolivia is scarce and unactualized. Therefore, their population becomes neglected in both, the Bolivian Lowlands and Highlands. Nutritional parameters in children, a vulnerable population, were determined. Clinical information, anthropometrical parameters like weight and height; haematological and biochemical indicators and examination of parasites in stool were collected from 120 children from rural Taraco (Highlands) and 96 from semi-urban Caranavi (Lowlands), both predominantly of Aymara indigenous ethnicity.

Questions

- ◆ What is the nutritional situation in children from the Bolivian Lowlands and Highlands?
- ◆ Do the environmental and socio-demographic conditions affect the nutritional status in children from the Bolivian Lowlands and Highlands?

Main findings

Overweight and obesity were notably prevalent in scholar children from the Bolivian Lowlands.

A surprising prevalence of 41% over-weighted and obese children aged 6 to 12 years, was found in the community of Caranavi, in the Lowlands. In contrast, only 3% of children included in the study were undernourished.

Severe nutritional concerns, including undernourishment, hypovitaminosis D and anaemia were found in scholar children from the Bolivian Highlands.

Around 11% of scholar children aged 6 to 12 of Taraco community in the Highlands, were undernourished, and 6,7% were stunted. In contrast to Caranavi, only 8% were obese or over-weighted children. Around 60% of all children showed insufficient vitamin D levels, but similar prevalence was also found in the Lowlands. As for anaemia, a very high prevalence of 74% was found. Socio-demographical and environmental conditions played an essential role in children's nutritional situation

In Taraco, a community with dry soil, low vegetation and a cold climate (typical from a 4000 masl area in the Highlands); undernourishment, anaemia and hypovitaminosis D were found. Moreover, two species of intestinal parasites that usually indicate poor hygienic conditions were identified, *Hymenolepis nana* (*H. nana*) in 78% and *Entamoeba coli* (*E. coli*) in 21% of all stool samples, confirming the precarious sanitary circumstances.

Caranavi, a better developed semi-urban community in the lowlands, showed improved hygienic conditions in school-children, and the same parasites were less prevalent, 29% of *H. nana* and 8% of *E. coli*.

Discussion

The study of two different socio-demographical and environmental communities, allowed to identify some possible health consequences of the nutritional/ hygienic situation in schoolchildren.

On one side, a community in the Highlands, a dry environment, with very low availability of nutrients during the whole year ²²⁴, contributes to all the severe nutritional conditions found in children. Stunting is perhaps the best example to show that a situation of poor nutrition and repeated infections are key for a growth impairment, this parameter is measured as height-for-age following the WHO Child Growth Standards median ²²⁵. However, the growth curves used as standards are still not adapted to specific conditions (like high altitude), therefore, a “normal” growing curve remains unknown as a reference for high latitudes with low oxygen pressure.

While the current information and statistics about nutrition in Bolivia are scarce, most studies are focused in undernourishment, mostly in children. In contrast, little data exists regarding the other face of malnutrition: overweight and obesity that are highly increasing worldwide ⁵. The rural to urban development usually is followed by improvements of living conditions. Better sanitary systems, school infrastructure and housing condition help to prevent exposure to some diseases. Yet, this new improved condition is not always well controlled. This was clearly noticed in Caranavi, a semi-urban population that is starting to grow, and where all kinds of food with high calories, fat and sugar were introduced in the market at very low prices, accessible to low- income individuals that are still an important fraction of this community. We hypothesize this was a possible reason for the high increase of overweight and obesity found in Caranavi.

In this study, one more rural-lowland, subtropical community called “Aguas Turbias - La Reserva” was included, and same nutritional parameters were studied in children (unpublished data). However, expanding the study to one more location, permitted to identify an interesting correlation between urban development and increase of obesity, and, in contrast a lower urban development that goes in line with an increased undernourishment prevalence in children. The rural community of Taraco in the Highlands (RU-HL) showed a clear example of what epidemiologists call “the double-burden effect”, meaning that the presence of both undernourishment and overweight/obesity were prevalent, therefore, showing a coexistence of both types of malnutrition in the same place (refer to Figure 4). The “double-burden” is a phenomenon that is starting to be of concern in many other countries ³.

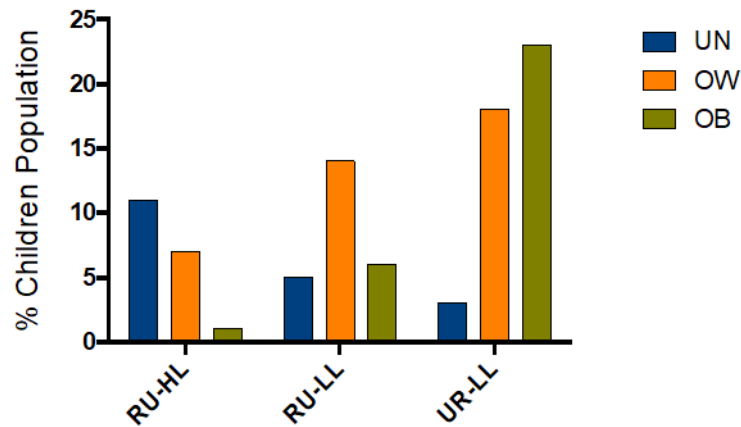


Figure 4. Prevalence of children that are unnourished (UN); overweight (OW) or obese (OB) in three different spots: Rural Highlands -Taraco (RU-HL); Rural Lowlands – Aguas Turbias/La Reserva (RU-LL) and Urban Lowlands-Caranavi (UR-LL). (Unpublished data).

Stool samples of children from the three previously mentioned communities were analysed by microscopy to determine the presence of intestinal parasites (unpublished data). The prevalence of intestinal parasites found in every community is depicted in Figure 5.

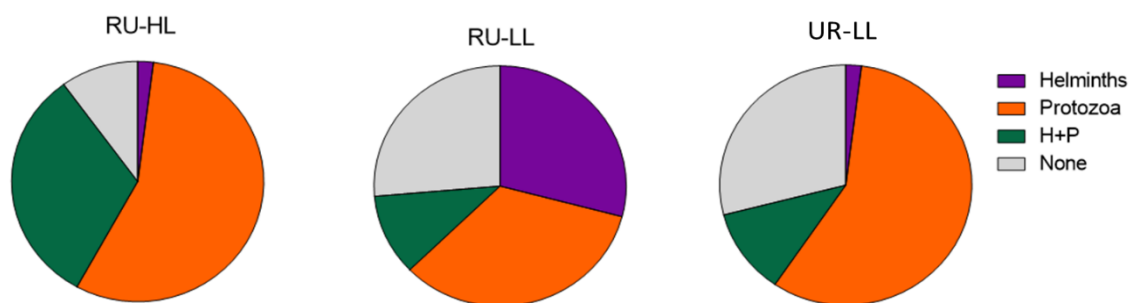


Figure 5. Prevalence of intestinal parasites in scholar children from: Taraco (RU-HL); Aguas Turbias/La Reserva (RU-LL) and Caranavi (UR-LL). Purple belongs to all children that only presented intestinal helminths; Orange is for children that only presented intestinal protozoa; Green belongs to children that presented both helminths and protozoa and Grey is the prevalence for uninfected children.

An important finding of the data above described is that the frequency of children with helminths (helminths alone + helminths and protozoa) is higher in Taraco (RU-HL) than in Caranavi (UR-LL) with 34% and 13,5% respectively, showing that high altitude settings, the poor hygiene and low sanitary conditions had a greater influence than a hostile and a high altitude geography that is usually not appealing for most of helminth species that prefer sub-tropical warm environments to fulfil their life cycle ²²⁶. Taken together, it can be hypothesized that helminths are capable to adapt to different environments and that an improvement of sanitary conditions and urbanization can decrease helminths prevalence and the constant risk of re-infections. An unexpected fact that is important to mention is the possible administration of anti-parasitic prophylactic treatment to children from Taraco and Caranavi few months before the study was performed, incident that might had an influence in the parasite prevalence obtained. However, it is relevant that even after chemoprophylaxis, the prevalence was still high, suggesting that re-infections are constant in these settings.

It is important to point out that one of the aims of this study was to evaluate a possible effect of intestinal parasitic infections on children's nutritional status. No correlation was found between intestinal parasites and nutritional parameters like anthropometry, Hb, total proteins, vitamin D, or zinc. Moreover, the deficient nutritional parameters showed in this study like undernourishment, hypovitaminosis D, anaemia, or lower albumin did not correlate with the presence of parasites. Constant re-infections in these children leading to asymptomatic chronicity, moderate parasitic load, and a combination of pathogenic and non-pathogenic species could account for this observation.

Intestinal parasites might not produce mortality and only severe cases or specific species will conduce to clinical symptoms ²²⁷. However, worm infections are still an important health problem that needs to be taken care of, not only due to their array of acute symptoms but also for their long term effects in infected children ²²⁸, or the possibility of previously described impairment on the efficiency of vaccination. Preventive chemotherapy is still the main prophylactic measurement in affected populations²²⁹. Moreover, health education, water treatment, improved hygienic and sanitary systems, and the reduction of consumption of contaminated water or soils are of vital importance, as suggested by our study.

Taken together, this study showed different nutritional deficiencies in the Highlands and Lowlands of Bolivia, and the influence of urban, sanitary and socio-economic conditions. (Explained in Figure 3).

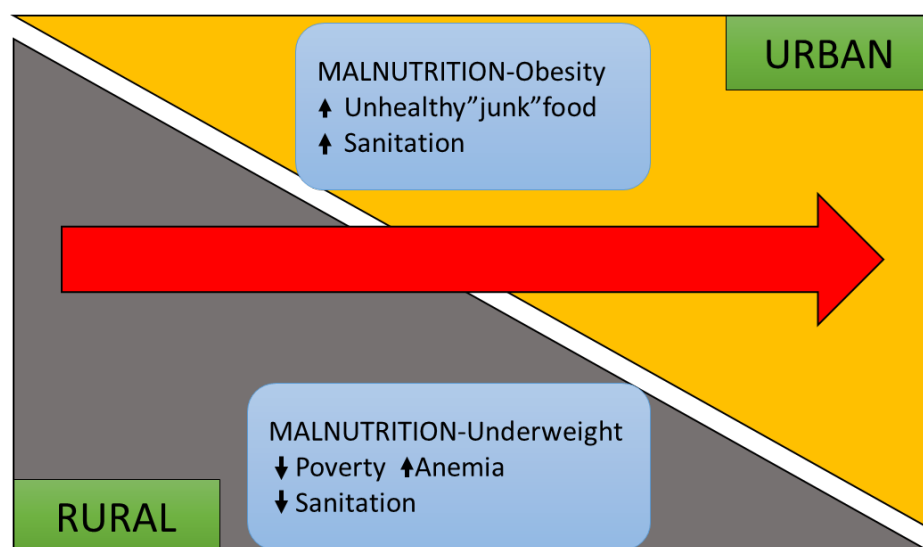


Figure 6. Scheme showing the main findings of the study in both Rural and Urban settings, where Malnutrition is a shared concern.

4.2. PAPER II: ATROPHY OF SKIN DRAINING LYMPH NODES PREDISPOSES FOR IMPAIRED IMMUNE RESPONSES TO SECONDARY INFECTION IN MICE WITH CHRONIC INTESTINAL NEMATODE INFECTION

Intestinal nematode infections are still a major global health concern, especially in rural communities of low-income countries ²²⁸. Apart from the important morbidity due to chronic infections produced by intestinal nematodes, it has been hypothesised that these infections may affect immunity to secondary infection and vaccination ²³⁰. However, results from studies on this subject are divergent.

This study investigated the effects of intestinal nematode infection on immune responses to secondary mycobacterial infection and the underlying mechanisms by which intestinal worms modulate immune responses to an infection distal to the site of worm infection.

Question

- ◆ How can chronic intestinal worm infections modulate immune responses to a secondary infection or vaccination in organs and tissues distal to the worm?

Main findings

Chronic *Heligmosomoides polygyrus* infection led to a paucity of lymphocytes in lymph nodes distal to the worm infection resulting in a reduced number of lymphocytes available to respond upon challenge with a secondary pathogen or vaccination. This finding relates to the so called “sink hypothesis” in which the mesenteric lymph nodes drain lymphocytes away from superficial skin-draining lymph nodes, which with time shrink.

Of importance, anti-helminthic treatment recovered LN cellularity, but time was required for the lymph node to regain cellularity and responsiveness to secondary antigen/infection.

Discussion

It has been shown that intestinal worm infection suppresses immune responses in conditions of allergy, gut inflammation and secondary infection and vaccination ²³¹. Alterations in Th2 cell differentiation and increased Treg cell suppressive functions have been some of several mechanisms that could be involved in this suppression ³⁰.

In this study, our results support the idea of lymphocyte paucity in the peripheral (not intestinal) secondary lymphoid organs in worm-infected mice. To start with, a reduced expansion of lymph nodes (LN) draining BCG injected footpad was found. We found no evidence for an increased frequency of regulatory T cells or higher levels of anti-inflammatory cytokines. The size of skin-draining LNs were smaller in worm-infected mice. However, an effective restoration of cellularity on skin-draining LN, and of the responses to BCG was reached after de-worming treatment.

An attempt to expand the lymphocyte pool was performed by frequent transfer of high numbers of lymphocytes to worm-infected mice, but no effect was seen on the LN cellularity from infected or control mice, concluding that a peripheral atrophy cannot be rescued providing “extra” lymphocytes. However, the lymphocyte pool was effectively expanded by

administration of recombinant IL-7 to worm-infected mice and helped to maintain size and cellularity of skin-draining LN, while the mesenteric LN did not expand more than in untreated worm infected mice. The function of IL-7 is of vital importance for lymphocytes development in the thymus and it also maintains survival and the homeostatic proliferation of naïve and memory T cells. Organogenesis of LN is also influenced by IL-7²³², supporting that IL-7 can indeed be involved in the regulation of lymphocyte pool expansion. However, a possible explanation of the lack of further expansion in the mLN indicates that expansion upon infection is also regulated.

4.3. PAPER III: ROLE OF HIF-1 IN TUBERCULOSIS AND DIABETES COMORBIDITY

Questions

- ◆ Is there a role of HIF in murine diabetes-tuberculosis comorbidity?

Main findings

Susceptibility to tuberculosis infection is increased in obese/diabetic mice. Bacterial load in lungs from db/db mice were enhanced as compared to C57BL/6 controls when measured 12 weeks after Mtb infection.

HIF-1 α -mediated responses were augmented during Mtb infection and diminished under hyperglycemia in BMM and in db/db Mtb-infected mice.

Particularly, *hif1a* and HIF-1 α -regulated responses were accumulated in mycobacteria-infected BMM. Moreover, reduced levels of HIF-1 α -regulated responses were found in Mtb and BCG-infected BMM under high glucose conditions.

Deferoxamine (DFO) a HIF chemical stabilizer, recovered impaired HIF-1 mediated responses in hyperglycemic conditions in vitro and reduced bacterial load in Mtb infected C57BL/6 mice; *hif1a* and HIF-1 α -regulated responses in mycobacteria-infected BMM were enhanced by DFO treatment. Moreover, when DFO was administrated in Mtb infected C57BL/6 controls, the levels of HIF-1 α -regulated lung transcripts increased, and a reduced bacterial load was found.

Using HIF-1 *hif1a^{fl/fl} lysm cre* BMM, the role of HIF-1 α in the responses of metabolic and immune genes during mycobacterial-infection of BMM was shown. An opposite effect was shown in *vh1^{fl/fl} lysm cre* BMM, a strain where HIF-1 α is stabilized.

Discussion

Here we hypothesized that an impairment of HIF-1 α -regulated immune and metabolic responses underlie TB-DM comorbidity.

A reduction of HIF-1 α -dependent immune and metabolic responses in hyperglycemic conditions was observed in BMM. Moreover, BMM incubated in high glucose conditions showed a diminished control of the intracellular growth of Mtb.

We also found increased bacterial load in a Mtb-infected diabetic murine model (db/db) when compared to non-diabetic controls. Previous data in other diabetic and obese models, like streptozotocin-induced diabetic models, ob/ob and sucrose-fed guinea pigs were reported as susceptible to Mtb infection^{233,234}. HIF-1 α -mediated responses in the lung were diminished in db/db as compared to WT mice. In contrast, our unpublished results in pre-diabetic HFD-fed mice with moderate hyperglycemia showed no increase in Mtb loads after 12 weeks of infection.

Perhaps the more explored explanation so far, is the negative effect of reactive oxygen species (ROS) through MGO generated by glycation of HIF-1 α or co-activator molecules¹⁹⁷. Several studies have shown that glycated albumin and methylglyoxal (a glycation intermediary) decrease the biological responses triggered when insulin binds to its receptor on the target cell. The molecular mechanisms that result in hampering HIF-1 functions remain to be studied.

To demonstrate the role of HIF-1 α in the reduced BMM responses during hyperglycemia, BMM with a conditional genomic deletion of HIF-1 α and VHL in myeloid cells were used. We observed that in contrast to WT BMM, *vh1^{fl/fl} lysm cre* BMM showed unimpaired HIF-1 α -dependent responses to mycobacterial infection in hyperglycemic as compared to euglycemic conditions.

In contrast *hif1 α ^{fl/fl} lysm cre* BMM showed dramatically diminished metabolic and immune responses to infection with mycobacteria. Therefore, HIF-1 α is central for the activation of the glycolytic pathway during the classical activation of macrophages. The control of intracellular Mtb infection was diminished in presence of hyperglycemia and was recovered in VHL-deficient BMM even in presence of high glucose concentrations.

Hif-1 α hydroxylation can also be blocked by chemical inhibition of PHD and, as a result, stabilize HIF-1 α . Deferoxamine (DFO) is an iron-chelator that binds free iron in a stable complex, preventing it from engaging in chemical reactions. PHD2 require Fe⁺⁺ as a cofactor and thereby it is inactivated by DFO. DFO is thus a HIF-1 α stabilizer. However, DFO is used to treat iron intoxication in transfusion patients²³⁵ and more recently to reverse the negative effect of hyperglycemia in wound healing during DM²³⁶ in experimental models and patients²³⁷. DFO could increase neovascularization and accelerate healing in these models. In relation, iron storage in the body has been reported to be associated with fat accumulation and type 2 DM. The excess iron causes oxidative stress through production of highly toxic oxygen radicals via the Fenton/Haber-Weiss reaction. As mentioned above, the increase production of reactive oxygen species is associated with the pathophysiology of DM. Thus, DFO might act at different levels to restore the altered HIF-1 α responses during hyperglycemia by impairing PHD activation or by reducing glycation of HIF-1 α or co-activator molecules. Based on these reasons and similar studies, DFO was selected as a HIF-1 α stabilizer in this study.

An effective response of BMM or mice with DFO *in vitro* was found. HIF-1 α and HIF-1 α -regulated immune and metabolic responses associated to the activation of the glycolytic pathway and the activation of macrophage oxidative burst was enhanced by incubation of mycobacteria infected BMM treatment with DFO. Therefore, DFO was tested *in vivo*. A clear effect was evident after DFO administration in Mtb infected C57BL/6 controls, the levels of HIF-1 α -regulated lung transcripts increased, and a reduced bacterial load was found. To explore the hypothesised effect of hyperglycemia and DFO, Mtb-infected diabetic mice were also DFO treated, but despite bacterial reduction in lungs was observed it did not reach significant levels (*Mann Whitney test p=0,06*) as compared to diabetic untreated controls. The

db/db mice showed hyperglycemia, reaching very high blood glucose concentrations that were maintained without significant fluctuations for three months. However, it is important to indicate that the db/db mice (as described in detail in methods section) also encompasses an array of altered metabolic and inflammatory responses that could diminish the effect of DFO. Taken these results together, we propose that and increased HIF-1 α function can improve control of infection with Mtb during DM, as schematically explained in Figure 7.

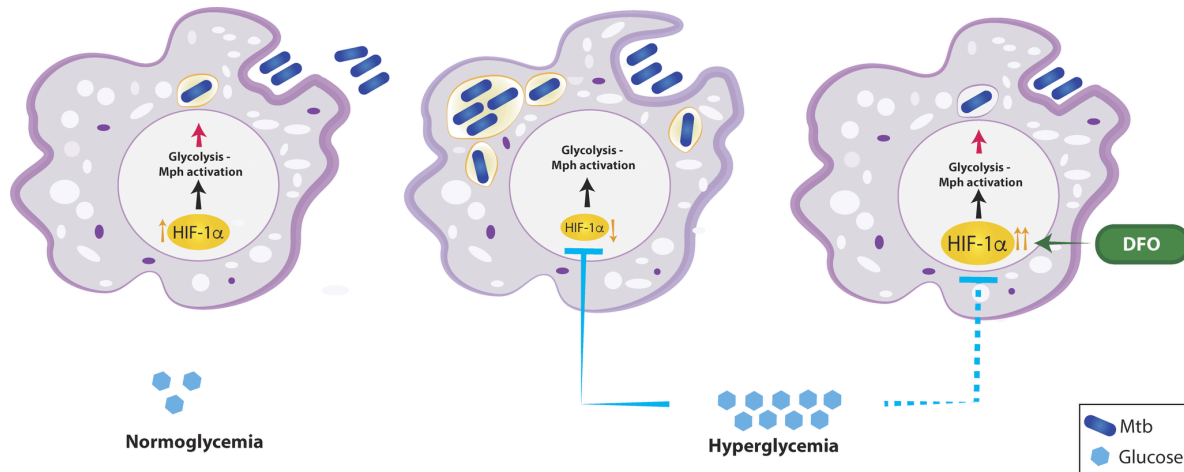


Figure 7. HIF-1 α has a protective role in Mtb infection that is affected under hyperglycemic conditions leading to higher Mtb infection. This effect is recovered with Deferoxamine (DFO), a HIF-1 α stabilizer, further controlling Mtb infection in macrophages.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, all the studies here presented, although a bit diverse, had a connective line which is the interaction between nutrition, infections and immune responses.

Here, the findings of these studies and their contribution to understand this interaction will be pointed and future perspectives briefly discussed.

The prevalence of overweight and obesity found in the Bolivian lowlands is a reminder of the importance of an increasing and challenging global health problem of this century. Moreover, the fact that the studied population were school children is even of higher concern. On the other hand, the described prevalence of undernourishment, stunting, anemia and an important deficiency in vitamin D, alerts about the persistency of health situations that were thought to be already solved, or at least decreased. The knowledge of a collective health problem helps the authorities of a community to, first be aware of the possible consequences, and second, to start improving nutritional and sanitary conditions with a major input on education. Intestinal parasitic infections are usually current in rural and semi-urban settings, as it was confirmed by showing an important prevalence. However, a correlation of infected children and nutritional deficiencies was not found. It is important to point out that this was a cross-sectional study, describing a “specific point time” situation. Therefore, a longitudinal study might be an improved setting in future. Moreover, an enlargement of the sample population and the addition of other communities with the same geographic situation could also improve the findings and comparisons for further analysis.

Immune responses were then explored in an effective murine model of intestinal helminth infection. The findings of the second study provided experimental marks about the immune modulation of helminth infections and their influence on secondary infections both systemically and skin-localized. Moreover, evidence of atrophic distal lymph nodes and accumulation of naïve lymphocytes into the draining lymph node showed a dynamic redistribution of the lymphocyte pool.

Finally, the startling finding of obesity and strong link with diabetes, together with an increasing incidence of TB-DM co-morbidity, inspired the third study. Furthermore, the interest of understanding possible involved mechanisms that could modulate metabolic and immune responses in both diseases, reinforced this idea. On this line, previous evidence about a possible role of HIF-1 α in both tuberculosis and diabetes seemed interesting to explore. The findings of TB susceptibility in diabetic mice supports the importance of TB-DM comorbidity. Moreover, evidence of a possible protective role of HIF-1 α linked to important immune and metabolic responses in TB and its impairment under hyperglycemic conditions, shows HIF-1 α as an important transcription factor to focus for deeper understanding of underlying mechanisms in TB-DM. Moreover, the effect of DFO as a HIF-1 α stabilizer that recovered these protective responses *in vivo*, as well as under hyperglycemic conditions *in vitro*, is of relevance because it supports further studies of HIF-1 α as a target and DFO as a therapeutic option.

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